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AUTORADIOGRAPHICAL LOCALIZATION OF NUCLEOSIDE TRANSPORT SITES AND ADENOSINE RECEPTORS IN HEART AND KIDNEY

by

© FIONA ELIZABETH PARKINSON

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **Autoradiographical Localization of Nucleoside Transport Sites and Adenosine Receptors in Heart and Kidney**, submitted by **Fiona Elizabeth Parkinson** in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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Date: July 7, 1989

To my parents,

who taught me to set goals,

and my husband,

who helps me to achieve them.

ABSTRACT

The distribution of nucleoside transport sites was investigated in rat and guinea pig heart and kidney using the autoradiographical ligand [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR). In guinea pig kidney, structures that appeared to be the glomeruli possessed a high density of binding sites; similar structures were not evident in rat kidney. In rat cardiac tissue, autoradiography of [^{3}H]NBMPR binding sites showed a uniform distribution, while guinea pig heart had a heterogeneous site distribution. Comparison of the areas of high site density with the radioimmunohistochemical distribution of von Willebrand Factor, an endothelial cell marker, indicated that endothelial cells accounted for the high density of [^{3}H]NBMPR binding sites.

Quantitative autoradiography was used to determine the binding constants for [^{3}H]NBMPR in these tissues. NBMPR binding sites in rat heart and the two binding areas in guinea pig heart all had high affinity (K_D values were 0.6-4.5 nM) for the ligand. Both binding areas in guinea pig heart contained sites with broad selectivity for nucleosides. The nucleoside transport inhibitor, lidoflazine, showed 8-fold selectivity for the endothelial cell binding component. This indicates that the two binding areas may possess different transporter subtypes.

The relative densities of adenosine A_1 receptors and nucleoside transport sites between cardiac myocytes and

atrioventricular conduction cells in guinea pig heart were investigated with the autoradiographical ligands 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine ([^{3}H]DPCPX), a selective A_1 receptor antagonist, and [^{3}H]NBMPR. Cardiac conduction cells were identified by histochemical detection of acetylcholinesterase. High resolution autoradiography indicated that conduction cells have a higher density of receptors, but an equal density of nucleoside transport sites to cardiac myocytes.

These studies indicate that the pronounced dromotropic effects of adenosine in guinea pig heart are correlated with a higher density of adenosine A_1 receptors in atrioventricular conduction cells. The transport capacity of these cells, as estimated by [^{3}H]NBMPR binding site density, is apparently not increased in proportion to A_1 receptors. The high density of transport sites associated with endothelial cells points to the key role of these cells in adenosine homeostasis in guinea pig heart.

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CHAPTER 1

INTRODUCTION

This chapter reviews the literature relevant to the research described in subsequent chapters. Most of the research presented here involved identifyication of nucleoside transport sites in heart tissue using the ^{3}H-labelled ligand, nitrobenzylthioinosine (NBMPR). A separate study investigated the adenosine A_1 receptor distribution between myocytes and conduction cells in heart. It is a premise of this thesis that through increased knowledge of nucleoside transport systems of heart, and greater understanding of the relationships between adenosine transporters and receptors in cardiac cells responsive to adenosine, it may be possible to potentiate particular actions of endogenous adenosine for therapeutic benefit. Therefore, in this chapter, a brief discussion of adenosine receptors will be presented first, followed by a description of the effects of adenosine on cells of the cardiovascular system. Nucleoside transport systems will then be described with emphasis on the transport inhibitor, NBMPR, and on differences in drug affinities among facilitated diffusion systems. Finally, the rationale for the present research will be given.

A. ADENOSINE RECEPTORS

The cardiovascular effects of adenosine occur subsequent to the binding of adenosine to membrane-bound receptors.

These receptors have been classified as A_1 and A_2 subtypes (Van Calker et al., 1979). In this original classification A_1 receptor stimulation was associated with inhibition of adenylate cyclase activity and A_2 receptors were associated with stimulation of adenylate cyclase. Typically, however, these receptors have not been classified on the basis of agonist effects on adenylate cyclase activity, but rather on rank order of potency of adenosine and analogues in effecting receptor-mediated responses (Bruns et al., 1987). A_1 receptors are defined as sites at which N^6-phenylisopropyladenosine is more potent than adenosine, which is more potent than 5'-N-ethylcarboxamidoadenosine. Additionally, A_1 receptors are stereoselective with the *R*-stereoisomer of N^6-phenylisopropyladenosine being more potent (about 10-40-fold) than the *S*-stereoisomer. In contrast, A_2 receptors show the reverse order of potencies (5'-N-ethylcarboxamidoadenosine > adenosine > N^6-phenylisopropyladenosine) and little (less than 10-fold) or no stereoselectivity for the *R*- and *S*-stereoisomers of N^6-phenylisopropyladenosine (Bruns et al., 1987).

Methylxanthines, such as aminophylline, theophylline, and caffeine, are antagonists at both A_1 and A_2 receptors. Currently, an active area of research is the development of compounds that are selective for each receptor subtype. Recently, 8-cyclopenyl-1,3-dipropylxanthine has been investigated and found to show selectivity for A_1 receptors.

Its potency has been reported as 500-700-fold greater at A_1 receptors than at A_2 receptors (Lee and Reddington, 1986; Lohse et al.,1987; Bruns et al., 1987). There are no known high affinity, selective antagonists for A_2 receptors at present.

B. CARDIOVASCULAR EFFECTS OF ADENOSINE

Effects of adenosine on the cardiovascular system were first described by Drury and Szent-Gyorgyi (1929), who reported that sinus bradycardia, delayed atrioventricular conduction, and increased coronary blood flow occurred in response to adenosine administration. Berne (1963) proposed that these actions constitute a regulatory function for adenosine. Under conditions of high metabolic activity or limited oxygen availability adenosine is released from myocardial cells. Depression of cardiac activity occurs in conjunction with coronary vasodilation, and thus oxygen supply is increased, while oxygen demand is decreased (Berne et al., 1987).

Vasodilation

Adenosine produces vasodilation in the vascular beds of many organs (Berne, 1986), including heart (Berne, 1980), brain (Berne et al., 1983), skeletal muscle (Berne et al., 1983), adipose tissue (Sollevi and Fredholm, 1981), intestine (Granger and Norris, 1980) and spleen (Schutz et al., 1983).

In contrast, adenosine causes vasoconstriction in kidney (Spielman et al., 1987).

Both *in vivo* and *in vitro*, adenosine produces vasodilation through a direct action on vascular smooth muscle cells. While it has been reported that, in cultured rat aortic smooth muscle cells, vasodilation is due to A_1 receptor stimulation of particulate guanylate cyclase and an increase in intracellular levels of cGMP (Kurtz, 1987), it is widely accepted that the receptors involved are of the A_2 subtype and activation of these receptors leads to adenylate cyclase stimulation and increased cAMP concentrations within the cell (Anand-Srivastava et al., 1982; Fredholm and Sollevi, 1986). While the receptors are different, the way in which adenosine produces vasodilation is analagous to the mechanism of beta-adrenoceptor agonist-induced vasodilation. The increase in intracellular cAMP activates cAMP-dependent protein kinase which then phosphorylates myosin light chain kinase. This reduces the binding of calmodulin-Ca^{++} with myosin light chain kinase and results in inhibition of phosphorylation of myosin and relaxation (Somylo and Somylo, 1986).

The contribution of endothelial cells, and of endothelium-derived relaxing factor to vasodilation produced by adenosine appears to be tissue and species dependent. While there are some examples of adenosine producing vasodilation in a partially endothelium-dependent manner

(Gordon and Martin, 1983; Frank and Bevan, 1983; Rubanyi and Vanhoutte, 1985; Yen et al., 1988), in most cases, vasodilation induced by adenosine is endothelium-independent (Furchcott, 1983; 1984; DeMey and Vanhoutte, 1982; Kennedy et al., 1985; McCormack et al., 1989).

Adenosine also produces vasodilation by another mechanism: by inhibiting transmitter release from the sympathetic nerves associated with blood vessels. Activation of presynaptic adenosine receptors (A_1 subtype) inhibits noradrenaline release and thus reduces $alpha_1$-adrenoceptor-mediated constriction of vascular smooth muscle (Fredholm and Sollevi, 1986).

Some coronary vasodilators, such as dipyridamole and dilazep, have been found to be inhibitors of nucleoside transport systems. The vasoactivity of these compounds has been attributed to the potentiation of adenosine-mediated vasodilation, resulting from inhibition of transport of adenosine into cells (Berne, 1983).

Negative Chronotropy

Adenosine produces a negative chronotropic effect on the sinoatrial node (Drury and Szent-Gyorgyi, 1929). West and Belardinelli (1985a) demonstrated that, in rabbit sinoatrial node, adenosine caused a dose-dependent decrease in rate and a shift in the site of the leading pacemaker. Under control conditions, sinoatrial nodal cells activate

first followed by adjacent cells of the right atrium; however, in the presence of adenosine the site of the leading pacemaker shifted so that cells of the right atrium were first to activate followed by sinus nodal cells (West and Belardinelli, 1985a).

In examination of the effects of adenosine on cardiac myocyte action potential parameters, it was found that adenosine decreased the rate of diastolic depolarization. This was similar to the effect of acetylcholine, but was blocked by aminophylline and not by atropine (West and Belardinelli, 1985b). The effects of both acetylcholine and adenosine on these cells is to increase potassium conductance and to produce hyperpolarization (Sperelakis, 1987). Adenosine acts on the same channels as acetylcholine, but the channels are activated by an A_1 receptor rather than a muscarinic receptor. A guanine nucleotide regulatory protein that is sensitive to pertussis toxin links the A_1 receptor stimulation to the potassium channel activation (Bohm et al., 1986). This A_1 receptor, although not associated with inhibition of adenylate cyclase, appears similar to A_1 receptors in other tissues (Stiles, 1986).

Negative Dromotropy

Adenosine slows the conduction of impulses through the atrioventricular node. Whether adenosine produces sinus block or atrioventricular block is species-dependent: guinea

pigs are more susceptible to atrioventricular block while cats, dogs and rabbits are more likely to show sinus block (Drury and Szent-Gyorgyi, 1929). Belardinelli et al. (1980) demonstrated that the conduction velocity between the atria and the ventricles is slowed in such a way that the atrium to His bundle interval is increased while the His bundle to ventricle interval is unchanged. Clemo and Belardinelli (1986) studied action potential parameters of cells along the conduction pathway and found that adenosine caused depression of amplitude, duration and rate of rise of atrioventricular cell action potentials. Adenosine shortened the duration of the action potentials of atrial cells and had no effect on action potentials of nodal-His, His bundle or ventricular cells. Thus, the increased atrium to His bundle conduction time was due to the action of adenosine on atrioventricular nodal cells.

While it is generally thought that the mechanism of adenosine action on atrioventricular nodal cells is the same as that described for sinoatrial nodal cells (Clemo and Belardinelli, 1986; Sperelakis, 1987), this has not been demonstrated.

Negative Inotropy: Atrial Myocardium

In atrial myocytes, adenosine decreases the duration of action potentials. This is the result of an increased potassium conductance, leading to faster repolarization of

cells (Belardinelli and Isenberg, 1983a). This effect is similar to that seen with acetylcholine administration. The mechanism of adenosine action is the same as that described for sinoatrial nodal cells: A_1 receptors activate potassium channels via guanine nucleotide regulatory proteins (Sperelakis, 1987).

Due to the decreased duration of action potentials, the Ca^{++} slow current of the plateau phase is attenuated. This leads to decreased intracellular Ca^{++} available for contraction, and results in a negative inotropic effect.

Negative Inotropy: Ventricular Myocardium (Anti-adrenergic Effect)

In contrast to the effects of adenosine on atrial cells, in ventricular myocytes adenosine has no effect on action potential parameters nor on basal contractility (Belardinelli and Isenberg, 1983b). The negative inotropic effect of adenosine in ventricular preparations is only apparent after a positive inotropic response has been obtained with catecholamines or forskolin (Belardinelli and Isenberg, 1983b; West et al., 1986; Martens et al,. 1986; Henrich et al., 1987). These agents cause increased intracellular cAMP concentrations by stimulating adenylate cyclase activity. Increased cAMP stimulates cAMP-dependent kinase, which phosphorylates Ca^{++} channel proteins and increases the probability of channel opening with depolarization

(Sperelakis, 1987). Thus, increased Ca^{++} is available for contraction. Adenosine, by decreasing cAMP concentrations, attenuates the increased Ca^{++} current and reduces the force of contractions.

Adenosine acts at A_1 receptors coupled to adenylate cyclase through the inhibitory guanine nucleotide regulatory protein (G_i) (Linden et al., 1985; Martens et al., 1987). The number of A_1 receptors in ventricular tissue is very small: estimates from crude membrane preparations were about 15 fmol/mg protein in rat (Linden et al., 1985; Martens et al., 1987) and 30.5 fmol/mg protein in bovine (Lohse et al., 1985).

Clinical Uses of Adenosine

Adenosine is useful in the treatment of some cardiovascular diseases. For example, bolus injection of adenosine has been used both as a diagnostic tool and for treatment in atrioventricular node reentrant tachycardias in adults and children (DiMarco et al., 1983; Overholt et al., 1988). Adenosine appears to act during the first pass of the bolus through the heart. Because the effects of adenosine are transient, increased concentrations or an alternative drug can be used in rapid succession.

The hypotensive effects of adenosine have also been used to clinical advantage. Sollevi et al. (1984) reported the use of adenosine infusion to produce controlled hypotension

during cerebral aneurysm surgery. Infusions of low adenosine concentrations may be useful to reduce afterload in low cardiac output conditions or to dilate preferentially coronary vessels in coronary artery disease (Sollevi et al., 1987).

Adenosine has an antiaggregatory effect on blood platelets. Another proposed use of adenosine is to preserve platelets in the extracorporeal circulation during cardiopulmonary bypass surgery (Sollevi et al., 1987).

Adenosine is currently being investigated for use as a cardioprotective agent subsequent to acute myocardial infarction. After myocardial infarction, reperfusion of the affected tissue is important, but often reperfusion injury occurs. This is characterized by swelling of endothelial cells and cardiac myocytes, disruption of plasma membranes, and inadequate blood flow through the microcirculation. Adenosine infusions have been demonstrated to be beneficial in reducing reperfusion injury (Olafsson et al., 1987). This is hypothesized to be due at least in part to inhibition of neutrophil function, since adenosine decreases neutrophil adherence and free radical release in vitro (Cronstein et al., 1983; 1986; Olafsson et al., 1987). In support of this hypothesis, Engler (1987) reported minimal reperfusion injury when acute myocardial infarctions were reperfused with neutrophil-depleted blood.

C. NUCLEOSIDE TRANSPORT

Adenosine and other nucleosides enter mammalian cells by passive diffusion, facilitated diffusion, or sodium gradient-dependent transport. Passive diffusion is a minor component of adenosine permeation due to the hydrophilic nature of the compound. Facilitated diffusion nucleoside transport systems have been extensively studied in human erythrocytes and other mammalian cells. The facilitated diffusion systems of these various cells exhibit differences which will be discussed in more detail below. Sodium gradient-dependent transport enables nucleosides to be concentrated within cells. This system was first described in vesicles from renal and intestinal epithelia (LeHir and Dubach, 1984; Schwenk et al., 1984; LeHir and Dubach, 1985).

Nucleoside Transport in Human Erythrocytes

Detailed studies of nucleoside transport have been performed with human erythrocytes. This transport system is characterized as a carrier mechanism that accepts physiological nucleosides and deoxynucleosides as well as many synthetic nucleosides (Paterson et al., 1983; Gati and Paterson, 1989). Transport of nucleosides is nonconcentrative, reversible and rapidly equilibrating across the membrane. The transport process exhibits saturation phenomena, competition between permeants, and inhibition by specific compounds (Paterson et al., 1983; Gati and Paterson, 1989).

Nucleoside Transport Inhibitors

Nucleoside transport in human erythrocytes (Cass et al., 1974), erythrocytes from some other species (Jarvis and Young, 1980), cardiac myocytes (Heaton and Clanachan, 1987; Clanachan et al., 1987), and cultured mouse S49 lymphoma cells (Paterson et al., 1983) is selectively inhibited by some nucleoside analogues, such as nitrobenzylthioinosine (NBMPR) and nitrobenzylthioguanosine (NBTGR), at low concentrations. High affinity binding of NBMPR to membranes from these cells correlates with inhibition of nucleoside transport (Cass et al., 1974; Jarvis et al., 1982a).

An important tool in nucleoside transport research is [^{3}H]NBMPR. This compound has been used as a binding probe to identify nucleoside transport sites in many tissues, including cardiac membranes (Williams et al., 1984), CNS membranes (Hammond and Clanachan, 1983; 1984; 1985; Geiger and Nagy, 1984; Nagy et al., 1985), erythrocytes (Hammond et al., 1981; Jarvis et al.,1982a; 1982b; 1983) and cardiac myocytes (Heaton and Clanachan, 1987; Clanachan et al., 1987). These studies demonstrated high affinity, saturable binding for [^{3}H]NBMPR that could be displaced by nucleosides, such as adenosine and uridine, and coronary vasodilators, such as dipyridamole and dilazep. In human erythrocytes, nucleoside-permeable sheep erythrocytes, and cardiac myocytes from rat and guinea pig, binding of NBMPR produced inhibition of nucleoside transport

(Jarvis et al., 1982b; Heaton and Clanachan, 1987; Clanachan et al., 1987). In addition, inhibition constants obtained for drug-induced inhibition of [^{3}H]NBMPR binding and inhibition of nucleoside transport were similar (Heaton and Clanachan, 1987; Clanachan et al., 1987). As a result of these studies [^{3}H]NBMPR has been regarded as a marker for equilibrative nucleoside transport systems in cardiac cells.

In heart, coronary vasodilators, such as dipyridamole, dilazep, hexobendine, and lidoflazine, competed with [^{3}H]NBMPR for high affinity binding sites, inhibited cellular accumulation of [^{3}H]adenosine, and potentiated the negative inotropic effects of adenosine (Williams et al., 1984). The therapeutic effects of these compounds are attributed to the inhibition of adenosine transport, in that they are thought to enhance and prolong the effects of adenosine at its extracellular receptors. In support of this, nucleoside transport inhibitors induce the same spectrum of activity in the cardiovascular system as adenosine: negative chronotropy, dromotropy, and inotropy, and coronary vasodilation.

NBMPR Binding Sites

The number of NBMPR binding sites in erythrocyte membranes varies among different species, but nucleoside translocation capacities (maximum rate of uridine influx divided by the maximum number of NBMPR binding sites per cell;

V_{max}/B_{max}) were similar across species (Jarvis et al., 1982a). This finding lent support to the concept that NBMPR binds to nucleoside transporters. In erythrocytes that lack nucleoside transport, high-affinity NBMPR binding is absent. Mutagenization of cultured S49 mouse lymphoma cells gave rise to an adenosine-resistant clone (AE_1) that did not show nucleoside transport or NBMPR binding (Cass et al., 1981). Thus, these two properties appeared to be intimately related.

Jarvis et al. (1982b) showed that NBMPR inhibited uridine influx in a competitive manner, but inhibited uridine efflux in a non-competitive manner. These results were taken as evidence that NBMPR binds to the outer face of the membrane and that the binding site and the nucleoside permeation site are identical or overlapping. Support for this model includes the finding that the dissociation constant (K_D value) for NBMPR binding is similar to the inhibition constant (K_i value) for inhibition of uridine influx, and that the K_i value for inhibition of NBMPR binding by uridine is similar to the Michaelis Menten constant (K_m value) for uridine influx (by equilibrium-exchange) (Jarvis et al., 1982b; 1983).

While the above discussion indicates a close association between the high-affinity NBMPR binding site and the nucleoside permeation site, this is not always true in cultured neoplastic cells. Many studies have reported nucleoside transport systems that are not inhibitable by low

concentrations of NBMPR.[1] These include Novikoff hepatoma cells (Plagemann and Wohlheuter, 1985) and Walker 256 carcinosarcoma cells (Paterson et al., 1985). NBMPR-sensitive and NBMPR-insensitive nucleoside transport exist together in cultured mouse leukemia L1210 cells (Belt, 1983) and HeLa cells (Dahlig-Harley et al., 1981). In studies with mutagenized S49 mouse lymphoma cells, one clone showed a partial loss of NBMPR-sensitive nucleoside transport without concomitant loss of NBMPR binding sites (Aronow et al., 1985), while other clones showed some loss of NBMPR binding sites without loss of transport capacity (Aronow et al., 1985; Cohen et al., 1985). These studies constitute genetic evidence that the NBMPR binding site is distinct from the nucleoside permeation site, and lend support to an allosteric interaction between these sites. More recently, nucleoside transport systems of low sensitivity to NBMPR in cells with high affinity NBMPR binding sites have been described. These were found in Novikoff UASJ-2.9, Morris 3924A and Reuber H-35 rat hepatoma cell lines (Paterson et al., 1987).

Nucleoside Transport System Heterogeneity

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[1] Facilitated diffusion nucleoside transport systems that are inhibited by low concentrations (< 10 nM) of NBMPR are termed "NBMPR-sensitive" and those that are only inhibited by higher concentrations (> 1 μM) or are not inhibited by NBMPR are termed "NBMPR-insensitive".

It will be apparent from the above discussion that all facilitated diffusion nucleoside transport systems are not identical. While high affinity NBMPR binding sites that are not associated with inhibition of nucleoside transport have not been identified in normal cells, there have been reports of NBMPR-insensitive transport. The first report of this dealt with rat erythrocytes (Jarvis and Young, 1986) in which both NBMPR-insensitive and -sensitive transport systems exist. The latter system was characterized as having higher affinity and lower translocation capacity for uridine than the NBMPR-insensitive system, and was not typical of transporters in other mammalian erythrocytes. NBMPR-insensitive nucleoside transport has also been described in rat cerebral cortical synaptosomes (Lee and Jarvis, 1988).

The use of dipyridamole has provided further evidence of nucleoside transporter heterogeneity. Hopkins and Goldie (1971) were the first to report that dipyridamole does not potentiate the effects of adenosine in rat tissue. It has since been demonstrated that dipyridamole has significantly lower affinity for nucleoside transport systems in rat tissues than in some other species, for example guinea pig. This was apparent for dipyridamole-induced inhibition of NBMPR binding, inhibition of nucleoside transport, and potentiation of adenosine-mediated effects (Williams et al., 1984; Clanachan et al., 1987). In addition, CNS membrane preparations from guinea pig and dog each demonstrated an

apparent single class of binding sites for NBMPR, but dipyridamole produced biphasic inhibition of NBMPR binding (Hammond and Clanachan, 1984; 1985). These results suggest the existence of two transporters that can be distinguished by dipyridamole and possibly by other transport inhibitors, but not by NBMPR. In these studies, the two forms of nucleoside transport were apparent within one organ and between species.

Another subtype of nucleoside transport that has yet to be fully characterized is based on substrate specificity. Thampy and Barnes (1983a; 1983b) described a nucleoside transport system that had high affinity (K_m = 13 μM) and selectivity for adenosine in neurons and a system with lower affinity (K_m = 370 μM) for adenosine and broader substrate specificity in cultured glial cells from embryonic chick brain. Geiger and co-workers (Geiger and Nagy, 1984; Geiger et al., 1985) have hypothesized that peripheral systems are characterized by broad substrate specificity, with K_m values for adenosine of 100 - 1000 μM, while neuronal systems are selective for adenosine, with K_m values of 1 - 10 μM. The adenosine-selective transporter of nervous tissue is proposed to be a salvage mechanism for adenosine released from nerves as a neurotransmitter.

Thus, it is evident that a diversity of facilitated diffusion nucleoside transport systems exist. To date these systems have been classified on the basis of dipyridamole

sensitivity, NBMPR sensitivity or substrate specificity.

D. RATIONALE

The studies described in this thesis used [^{3}H]NBMPR as a marker for NBMPR-sensitive transporters and investigated the heterogeneity of these transporters in terms of abundance or inhibitor affinity in rat and guinea pig hearts and kidneys.

These studies were intended to examine NBMPR-sensitive nucleoside transporters of normal tissues, and to look for differences among various cell types. The cell types of particular interest included coronary endothelial cells, ventricular myocytes and atrioventricular nodal cells. Endothelial cells are widely distributed and atrioventricular nodal cells are few in number; thus, histology and autoradiography were used to examine the different cell types in cardiac tissue sections rather than attempting to isolate and purify them. [^{3}H]NBMPR was used as the autoradiographical ligand because previous studies with rat and guinea pig ventricular myocytes demonstrated it to be a high-affinity ligand that produced inhibition of nucleoside transport (Heaton and Clanachan, 1987; Clanachan et al., 1987).

In a separate study, [^{3}H]DPCPX was used as an autoradiographical ligand for adenosine A_1 receptors in sections of guinea pig heart containing atrioventricular conduction cells. The greater effectiveness of adenosine at

conduction cells relative to other cardiac cells may be due to increased receptor density, decreased transporter density, or alterations in other factors such as coupling efficiency between receptors and transduction mechanisms or maximum rate of transport into cells. The abundance of A_1 receptors, identified with [^{3}H]DPCPX, and nucleoside transporters, localized with [^{3}H]NBMPR, in conduction cells relative to cardiac myocytes was determined to test whether either of these factors correlates with the increased sensitivity of conduction cells to adenosine.

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CHAPTER 2

NITROBENZYLTHIOINOSINE (NBMPR) BINDING SITES IN KIDNEY[2]

A. INTRODUCTION

Adenosine exerts various receptor-mediated regulatory effects in mammalian cells. Infusion of adenosine into renal arteries of rats produces vasoconstriction, decreased glomerular filtration rates, inhibition of renin release and inhibition of sodium and fluid excretion (Spielman and Thompson, 1982). Similar effects were observed in the presence of dipyridamole, an inhibitor of facilitated diffusion nucleoside transport; these effects were reversed in the presence of theophylline, an antagonist at adenosine receptors (Arend et al., 1985). This suggests that dipyridamole potentiates the effects of endogenous adenosine. Nitrobenzylthioinosine (NBMPR) is a potent nucleoside transport inhibitor in many mammalian cell types (Clanachan et al., 1987; Jarvis et al., 1982). In these cells, binding of NBMPR has been used extensively to enumerate and characterize facilitated diffusion nucleoside transport systems (Clanachan et al., 1987; Jarvis et al., 1982).

The present experiments were designed to compare NBMPR binding parameters in rat and guinea pig renal preparations and to investigate the distribution of binding sites in these

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[2] A version of this chapter has been published. Parkinson, F.E. and Clanachan, A.S. 1987. Proc. West. Pharmacol. Soc. 30:75-77.

tissues.

B. METHODS

To prepare membrane protein, kidneys were minced, then homogenized (Polytron setting 6 for 10 sec) in 20 vol (w/v) of ice-cold sucrose (0.32 M). The homogenates were centrifuged at 1000 g for 10 min; then the supernatants were centrifuged at 20,000 g for 20 min. The resulting pellets were resuspended in Tris-HCl buffer (50 mM), pH 7.4, to make a final protein concentration of 0.75-1.0 mg/ml as determined by the method of Lowry et al. (1951).

Equilibrium binding assays (0.5 ml volume) contained [^{3}H]NBMPR (0.01-5 nM) in the presence (non-specific binding component) or absence (total binding) of dilazep (100 μM) or dipyridamole (30 μM for guinea pig preparations or 100 μM for rat preparations), and were initiated by the addition of membrane protein (0.3-0.4 mg/ml). Incubations of 30 min at 22-23^{0}C were terminated by filtration (Whatman GF/B filters washed twice with 2 ml ice-cold buffer). Filter-associated tritium radioactivity was determined by liquid scintillation spectrometry. Site-specific binding was represented by the difference between total and non-specific binding.

Kidneys were cut in half dorsoventrally, embedded in tragacanth gum and OCT Compound, and rapidly frozen (40 sec) in isopentane cooled with liquid nitrogen. Frozen kidneys were stored (<10 days) at -70^{0}C. Cryostat sections (10 μm)

were cut and put on subbed microscope slides. The sections were kept in a desiccator at 4°C overnight, then frozen at -70°C for up to one week.

Equilibrium binding assays (12 ml volume) were performed with the slide-mounted tissue sections. A 5 min preincubation in phosphate-buffered saline (PBS) (4°C) was followed by a 30 min incubation at 22-23°C with [^{3}H]NBMPR (0.025-10 nM) in the presence or absence of dilazep or dipyridamole (same as for membrane preparations). The tissues were fixed for 10 min by adding 1 ml of formaldehyde (5.6%) to each incubation medium. The sections were washed twice for 5 min with buffer (4°C) then rinsed quickly three times with distilled water (4°C). The sections were blown dry (4°C), stored in a desiccator overnight, then apposed to LKB ultrofilm in X-ray cassettes and stored (4°C) in the dark for three weeks.

C. RESULTS

Site-specific binding of [^{3}H]NBMPR was apparent in both rat (n=5) and guinea pig (n=5) renal membrane preparations. Binding constants, K_D (geometric mean and 95% confidence interval) and B_{max} (arithmetic mean ± standard error of the mean), determined by Scatchard analyses were 0.13 (0.05-0.37) nM and 534 ± 79 fmol/mg protein, respectively, for guinea pig and 0.05 (0.03-0.06) nM and 107 ± 11 fmol/mg protein, respectively, for rat renal membrane preparations.

Due to the high affinity of [^{3}H]NBMPR and low binding site density in rat membranes, it should be recognized that some data points were at the limits of sensitivity for the detection of tritium by liquid scintillation spectrometry.

Autoradiographical localization revealed non-uniform [^{3}H]NBMPR binding site distributions (Fig. 4.1) in guinea pig and rat kidney. Sections from both species exhibited greater binding site density in cortex than in medulla. Guinea pig sections had notably higher binding site density overall than was apparent in rat sections. The most striking feature of the autoradiograms of guinea pig kidney was the very high density of [^{3}H]NBMPR binding in structures that appeared to be glomeruli. These areas of high binding site density were not observed in the rat kidney autoradiograms.

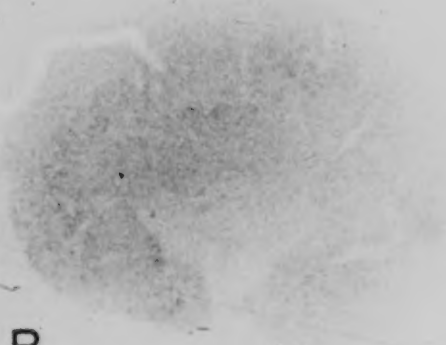
D. DISCUSSION

The major findings of these experiments were that [^{3}H]NBMPR binding was greater overall in guinea pig than in rat kidney sections; in both species the binding site density was greater in the renal cortex than in the medulla. Also, in guinea pig sections, but not in rat sections, the glomeruli appeared to have a very high [^{3}H]NBMPR binding site density.

In a previous study, autoradiography revealed a markedly heterogeneous [^{3}H]NBMPR binding site density in guinea pig, but not rat, cardiac sections (Parkinson and Clanachan, 1986). The high density component was apparently derived from the

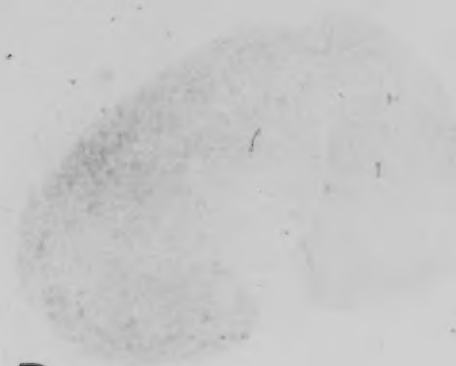
FIGURE 2.1: Autoradiographical localization of [^{3}H]NBMPR binding sites in guinea pig (A, B) and rat (C, D) kidney sections (10 μm). Equilibrium binding assays were performed with [^{3}H]NBMPR (3 nM) in the absence (total binding; A, C) or presence (non-specific binding component) of dipyridamole (30 μM, B; 100 μM, D).

A



B

C



D

coronary vasculature (Parkinson and Clanachan, 1986). As yet, it is unclear which cell types are responsible for the high density binding components in the glomeruli and coronary vasculature of guinea pigs, but endothelial cells would seem likely candidates.

Sodium gradient-dependent transport of nucleosides has been reported in rat renal brush border vesicles (LeHir and Dubach, 1984; 1985a; 1985b). This is described as a concentrative transport mechanism that is not inhibited by NBMPR and is distinct from the facilitated diffusion system described for other mammalian cells (Clanachan et al., 1987). Although NBMPR has been shown to be a nucleoside transport inhibitor in guinea pig and rat cardiac myocytes (Clanachan et al., 1987) and erythrocytes (Jarvis et al., 1982), further studies are required to determine whether it inhibits nucleoside transport in the various cellular components of kidneys from these species.

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CHAPTER 3

HETEROGENEITY OF NUCLEOSIDE TRANSPORT INHIBITORY SITES IN HEART: A QUANTITATIVE AUTORADIOGRAPHICAL ANALYSIS[3]

A. INTRODUCTION

Adenosine is an endogenous nucleoside that exerts important regulatory effects on cardiovascular function. At least two cell surface adenosine receptors have been recognized; stimulation of the A_1 adenosine receptor subtype results in negative inotropic, chronotropic and dromotropic effects in the heart, while A_2 adenosine receptor activation causes vascular smooth muscle relaxation. The coronary vasculature is particularly sensitive to adenosine and it has been proposed that adenosine, released from cells during periods of hypoxia and high metabolic demand, could be an important link in regulating coronary blood flow and oxygen delivery to cardiac cells (Berne, 1980). In addition, adenosine may serve as an endogenous regulator of cardiac excitability and contractility (Dobson and Fenton, 1983). The cardiovascular effects of adenosine have been exploited therapeutically for the management of supraventricular tachycardia (DiMarco et al., 1983) and for the production of controlled hypotension during anaesthesia (Sollevi et al., 1984).

Adenosine is rapidly cleared from the vicinity of its

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[3] A version of this chapter has been published. Parkinson, F.E. and Clanachan, A.S. 1989. Br. J. Pharmacol. 97:361-370.

cell surface receptors by rapid uptake systems consisting of membrane-located nucleoside transport mechanisms and subsequent intracellular metabolism. Facilitated diffusion nucleoside transport systems have been characterized in many cell types, including cardiac myocytes, and are inhibitable by pharmacologically diverse groups of drugs including coronary vasodilators, calcium channel blockers and benzodiazepines (Williams et al., 1984; Hammond et al., 1985; Ford et al., 1985). Many of the coronary vasodilator drugs, for example dipyridamole and dilazep, are believed to exert some of their therapeutic effects by interacting with transport inhibitory sites associated with the nucleoside transport mechanism. In this way, adenosine influx is inhibited and its levels close to adenosine receptor sites are sustained leading to potentiation and prolongation of adenosine-mediated effects.

At present, it has not been determined where transport inhibitors bind on nucleoside transport complexes. Jarvis and Young (1982) proposed a model where inhibitors bound to the permeation site to produce inhibition of nucleoside transport; however, Koren et al. (1983) proposed that transport inhibition may result from an allosteric interaction between the inhibitor binding site and the nucleoside transport site. In the absence of definitive knowledge of the specific site of interaction of NBMPR with the transport protein, we have adopted the term "nucleoside transport inhibitory sites" to refer to compounds that bind to sites,

occupancy of which inhibits nucleoside permeation.

The study of drug interactions with nucleoside transport mechanisms has been facilitated by the use of a high affinity probe, [^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR) (Cass et al., 1974). NBMPR and congeners bind with high affinity and selectivity to membrane-located sites in many cell types including erythrocytes from various species and cardiac myocytes. Occupation of these sites inhibits nucleoside transport and inhibition constants of drugs for the inhibition of [^{3}H]NBMPR binding is predictive of their transport inhibitory activity (Williams et al., 1984).

Subtypes of facilitated diffusion nucleoside transport systems have recently been identified; systems of high or low sensitivity to NBMPR are present in several cloned and uncloned lines of cultured cells (Paterson et al., 1987) and these systems may coexist in some cell types. Systems of high and low sensitivity to dipyridamole are detectable in guinea pig and rat myocytes, respectively (Heaton and Clanachan, 1987; Clanachan et al., 1987). However, it is not known if subtypes exist either in the same cell or in the different cellular elements within heart. The discovery of regional differences in transporter distribution or regional subtypes differing in drug affinity would have important implications in the understanding of the mechanisms of adenosine efflux (production) and influx (inactivation). Selective inhibition of nucleoside transporter subtypes might allow the selective modulation of the regulatory actions of adenosine.

In this study we have determined NBMPR binding constants in different regions of guinea pig and rat heart using quantitative autoradiography. This procedure also permitted the measurement of the affinity of dipyridamole for NBMPR sites in order to examine the potential heterogeneity of transport inhibitory sites in these tissues.

B. METHODS

Tissue Preparation

Sprague-Dawley rats (males, 200-400 g) and Hartley guinea pigs (males, 200-400 g) were killed by a blow to the head followed by exsanguination. The hearts were quickly removed and the ventricles were embedded in sectioning media (20 parts OCT Compound: 10 parts distilled water: 7 parts tragacanth gum), frozen in isopentane cooled with liquid nitrogen and stored at -70°C for up to two weeks. Transverse sections (10 μm) were cut (-22°C) with a cryostat (International Equipment Company model CTI), mounted onto gelatin-coated slides, desiccated overnight at 4°C and then used immediately or stored at -70°C for up to two weeks.

Biochemical Analysis of [^{3}H]NBMPR Binding to Tissue Sections

The characteristics of [^{3}H]NBMPR binding to tissue sections on microscope slides were determined for the purpose of subsequent autoradiographical studies. Two microscope slides with two (guinea pig) or three (rat) sections per slide were used for each determination. The sections were preincubated for 5 min in ice-cold phosphate-buffered saline

(PBS; NaCl 137 mM, Na_2HPO_4 6 mM, KCl 2.7 mM, KH_2PO_4 1.5 mM, $CaCl_2$ 0.9 mM, $MgCl_2$ 0.5 mM; pH 7.4), then incubated with graded concentrations of [^{3}H]NBMPR (0.05-10.0 nM) for 30 min at 20°C. Fixation of the tissue sections resulted from the addition of formalin to the incubation medium (final conc. 0.4%) for 10 min. This method of fixation was found not to alter the total or non-specific binding. The tissue sections were washed twice for 5 min in ice-cold PBS then quickly rinsed three times in ice-cold distilled water. Non-specific binding of [^{3}H]NBMPR was determined using alternate slides processed as above, but incubated with [^{3}H]NBMPR in the presence of dipyridamole (30 μM for guinea pig sections or 100 μM for rat sections) or dilazep (100 μM).

Dipyridamole competition studies for guinea pig sections were performed in the presence of 2.8-3.2 nM [^{3}H]NBMPR and graded concentrations of dipyridamole (1 nM-30 μM). For rat sections, [^{3}H]NBMPR was used at concentrations of 0.7-0.9 nM, dipyridamole concentrations ranged from 0.1 to 100 μM, and the non-specific binding was determined using 100 μM dilazep. Sections were wiped from the slides with Whatman GF/B filters and the radioactivity was counted (Beckman LS6800). For every six slides used as above, one was scraped with a razor blade and the tissue sections were assayed for protein content using the method of Lowry et al. (1951) with bovine serum albumin as the standard. The data were analyzed to yield estimates of the dissociation constants (K_D; nM), maximal numbers of binding sites (B_{max}; fmol/mg protein) and dipyridamole

inhibition constants (K_i; nM or μM).

Autoradiographical Analysis of [^{3}H]NBMPR Binding to Tissue Sections

Equilibrium binding assays and dipyridamole competition assays were performed as above with guinea pig and rat slide-mounted tissue sections. Instead of wiping the sections from the slides, they were blown dry with cold air in a cold room (4°C) and desiccated overnight. In addition, competition assays with inosine, a transporter permeant, were performed with guinea pig sections in the presence of 3.2 nM [^{3}H]NBMPR and graded concentrations of inosine (3 μM- 3 mM). Tissue sections were apposed to Ultrofilm ^{3}H in x-ray cassettes and stored in the dark at 4°C for three weeks. Included with each film were two sets of 6 standards described below. The films were developed using Microdol X Developer for 10 min, rinsed with distilled water, fixed with Kodak Fixer for 5 min, and washed in running tap water for 20 min, all at 20°C.

Preparation of Standards. Radioactive standards were required to enable quantitation of autoradiograms; cardiac tissue was used in order to resemble the experimental sections as closely as possible, particularly in terms of quenching radioactivity. Ventricular tissue was minced into 10 mg pieces, homogenized in 20 volumes (w/v) of ice-cold 0.32 M sucrose (Polytron, setting 5 for 20-30 s), filtered through 250 μm mesh then centrifuged for 10 min at 20,000 g. Graded concentrations of a nonvolatile tritiated compound, [^{3}H]inulin, were thoroughly mixed with the ventricular paste to make six standard [^{3}H]

concentrations (Unnerstall et al., 1981). These were frozen and 10 μm sections were cut and mounted on microscope slides in a similar manner to the ventricular tissue sections. Samples from each standard were dried to constant weight, dissolved in NCS tissue solubilizer (300 μl), neutralized with glacial acetic acid (9 μl) and dpm/mg dry weight was determined for each standard by liquid scintillation spectrometry.

Small samples of guinea pig heart were dried to constant weight, solubilized in 1N NaOH and protein concentrations were determined by the method of Lowry et al. (1951) to determine mg protein/mg dry weight of heart tissue.

Quantitation of Autoradiograms

A computer image analysis system (Imageplus, Scientific MicroPrograms) was used to determine optical densities of different areas of the autoradiograms (high and low [^{3}H]NBMPR binding site density components in guinea pig sections and low density component in rat cardiac sections). Each experimental condition consisted of a minimum of eight tissue sections. Autoradiograms were coded and analyzed in random order by an individual who did not have access to the experimental condition code.

In order to convert the computer-derived optical density to fmol NBMPR bound/mg protein, a standard curve (ln dpm/mg dry weight versus ln optical density) was prepared for each sheet of Ultrofilm ^{3}H used, by the method of Unnerstall et al. (1982). Experimental values were converted to fmol NBMPR

bound/mg protein using the specific activity of the ligand and the value of mg dry weight/mg protein (determined as outlined above).

Von Willebrand Factor Radioimmunocytochemistry

Von Willebrand Factor is synthesized by endothelial cells and is a reliable marker for these cells (Jaffe, 1984; McComb et al., 1982). Immunoreagents for von Willebrand Factor were used in conjunction with [^{3}H]biotin to localize endothelial cells by autoradiography using a modification of the method described by Hunt and Mantyh (1984). Guinea pig ventricular sections (10 μm) were preincubated for 5 min in ice-cold PBS. This was followed by 10 min in 0.4% formaldehyde in ice-cold PBS and two subsequent 5 min washes in ice-cold PBS. Sections were then incubated in presoak buffer (5% bovine serum albumin (BSA), 0.05% NaN_3, 0.3% Triton X100 in PBS) for one hour at 20°C. Washes, at this and later steps, were performed with 1% BSA and 0.05% NaN_3 in PBS, 2 x 5 min at 20°C. Sections were then incubated with the primary antibody, rabbit polyclonal antibody to human von Willebrand Factor (1:1600 dilution in 1% BSA, 0.05% NaN_3, 0.3% Triton X 100 in PBS), for 1 hr at 20°C. Sections were washed, then incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (1:400 dilution in the same buffer used for primary antibody), for 1 hr at 20°C. Sections were washed again then incubated with [^{3}H]biotin (15 μCi) and avidin (10 μg) (previously mixed for 20 min at 20°C in 1.5 ml 1% BSA, diluted to 30 ml) for 20 min at 20°C. Five washes of 10 min each were performed using

the same wash buffer as previously. Sections were then blown dry, at 4°C, desiccated and apposed to Ultrofilm ^{3}H.

Specific binding to von Willebrand Factor was determined by the difference between sections incubated with and without the primary antibody; non-specific binding of avidin-[^{3}H]biotin complex was determined from sections not exposed to primary or secondary antibodies.

Materials

Compounds and reagents for these studies were obtained from the following sources: [G-^{3}H]nitrobenzylthioinosine (sp. act. 37 Ci/mmol), from Moravek Biochemicals, CA, and repurified as necessary before use; dipyridamole and avidin, from Sigma Chemical Co., St. Louis, MO; [^{3}H]inulin (sp. act. 3 Ci/mmol), from Amersham Canada Ltd., Oakville, Ont.; α-[8,9-^{3}H(N)]-biotin (sp. act. 33 Ci/mmol), from New England Nuclear, Boston, MA; rabbit polyclonal anti-human von Willebrand Factor, biotinylated anti-rabbit IgG and horseradish peroxidase-avidin D, from Dimension Laboratories Inc., Mississauga, Ont.; Ultrofilm ^{3}H, from Fisher Scientific, Edmonton, Alta; OCT Compound from Canlab, Edmonton, Alta. Dilazep was donated by Hoffman-LaRoche.

C. RESULTS

Biochemical Analysis of [^{3}H]NBMPR Binding to Tissue Sections

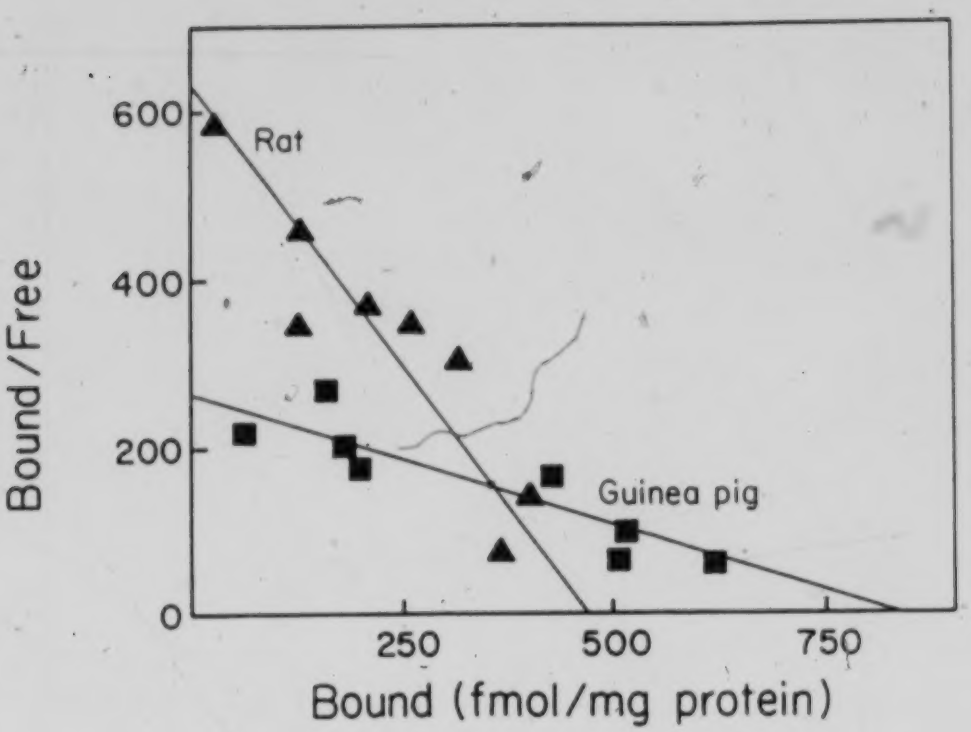
Site-specific binding of [^{3}H]NBMPR to slide-mounted sections of guinea pig and rat ventricles was saturable. Scatchard plots were linear (Fig. 3.1) and gave NBMPR binding

constants, K_D (geometric mean and 95% confidence interval) and Bmax ± s.e. mean, of 3.2 (1.4-7.1) nM and 931 ± 176 fmol/mg protein, respectively, for guinea pig (n=4) and 0.7 (0.5-1.0) nM and 419 ± 60 fmol/mg protein, respectively, for rat (n=3). These values are comparable to those observed in ventricular myocytes (Clanachan et al., 1987) and in crude ventricular membranes (Williams et al., 1984) prepared from these species. To ensure that the binding in ventricular sections involved the same site that had been characterized previously in myocytes and membrane preparations, we evaluated the affinity of dipyridamole in competition experiments with [^{3}H]NBMPR. The K_i values obtained were 70 nM for guinea pig and 3 μM for rat and the Hill coefficients were 0.81 and 0.91 for guinea pig and rat ventricular sections, respectively (Fig. 3.1).

Autoradiographical Localization

Autoradiography was performed to localize the total and non-specific [^{3}H]NBMPR binding components in guinea pig and rat ventricular sections. The difference between the total binding and non-specific binding autoradiograms represents site-specific binding. In guinea pig tissue sections, the specific binding had a non-uniform distribution which consisted of high and low density components (Fig. 3.2A,B). The high density binding component appeared to be associated with the coronary vasculature and endocardial lining. In contrast, a uniform distribution was evident in the total binding and non-specific binding autoradiograms for rat

FIGURE 3.1: Analysis of [^{3}H]NBMPR binding to guinea pig (■) and rat (▲) cardiac sections. Sections were wiped from microscope slides and associated tritium was determined by scintillation spectrophotometry. Upper panel: Scatchard analysis of specific binding. Abscissa: [^{3}H]NBMPR bound (fmol/mg protein). Ordinate: bound [^{3}H]NBMPR (fmol/mg protein)/free [^{3}H]NBMPR (nM). Best fit regression lines were drawn through the points of single representative experiments. Lower panel: Concentration dependent inhibition by dipyridamole of specific [^{3}H]NBMPR binding. Abscissa: $-\log_{10}$ [dipyridamole] M. Ordinate: [^{3}H]NBMPR bound (% control in the absence of dipyridamole). Data points are from single representative experiments.


600
400
200
0
Bound/Free
Rat
Guinea pig
250
500
750
Bound (fmol/mg protein)

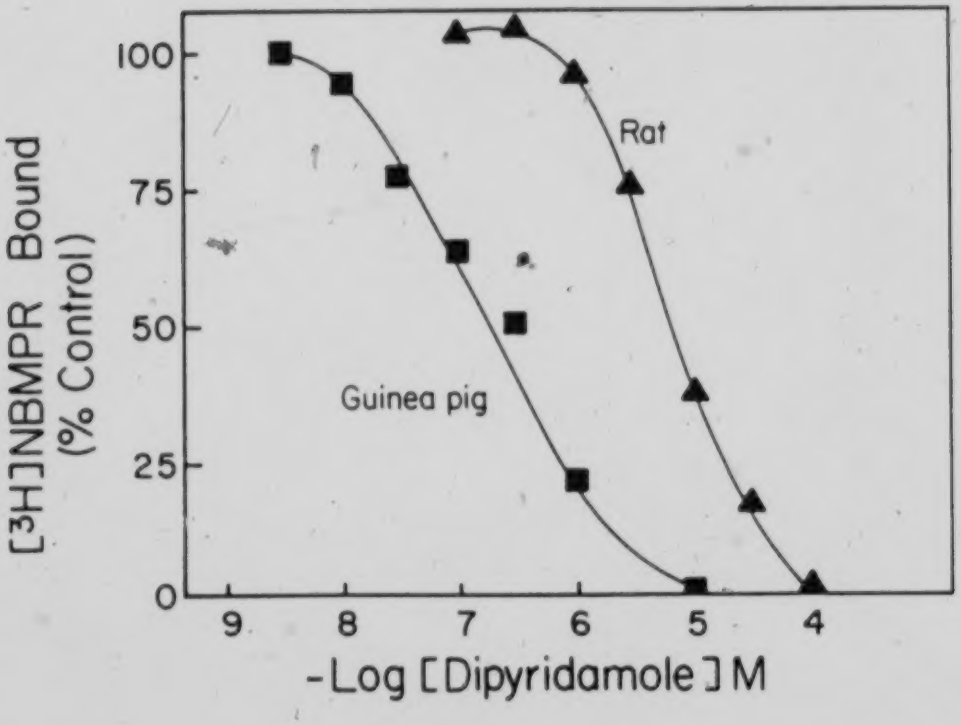
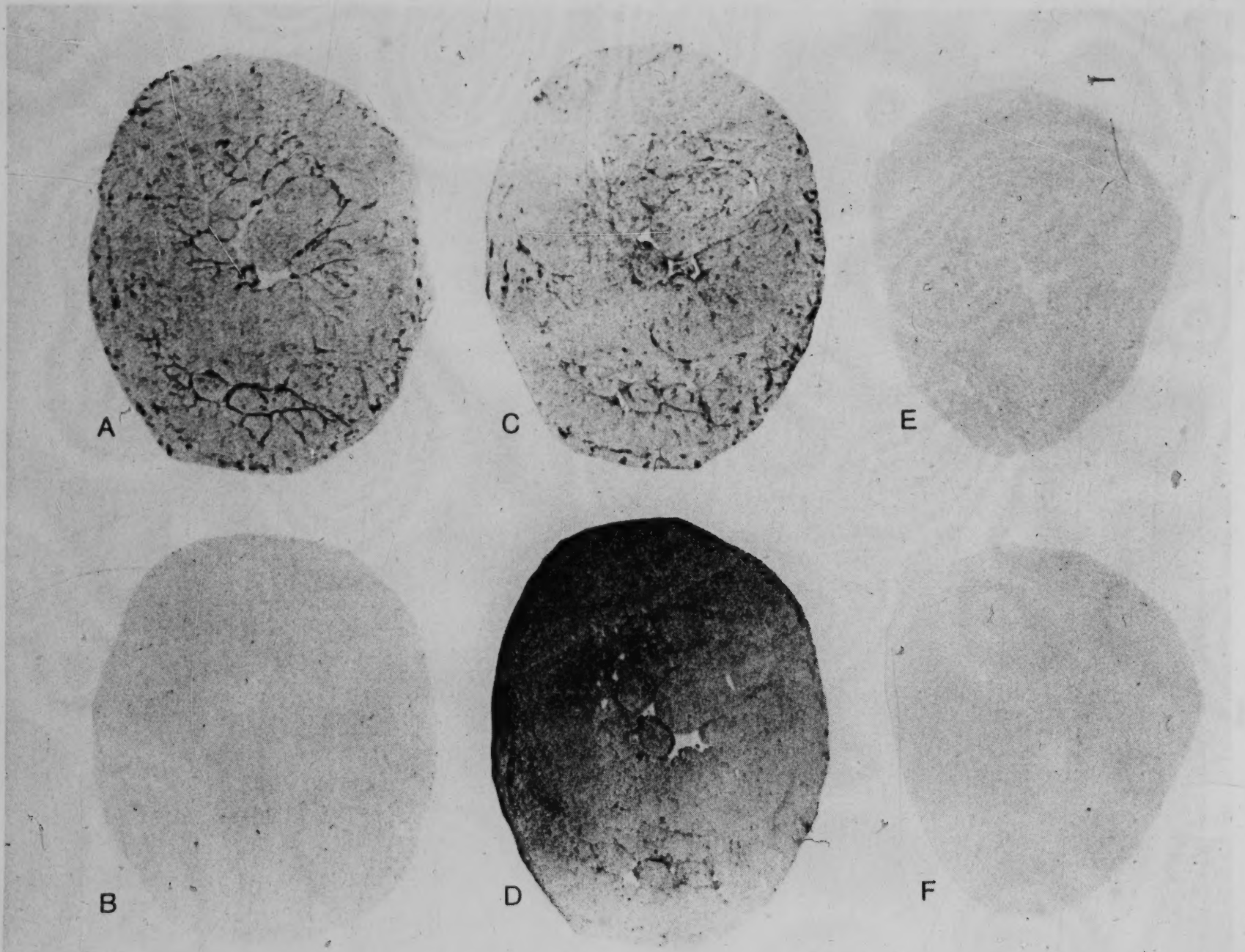

100
75
50
25
0
[3H]NBMPR Bound
(% Control)
Rat
Guinea pig
9
8
7
6
5
4
-Log [Dipyridamole] M

FIGURE 3.2: Autoradiographical localization of [^{3}H]NBMPR binding sites in guinea pig cardiac sections (A,B; 8 nM) compared to von Willebrand Factor (C,D) and [^{3}H]NBMPR binding sites in rat cardiac sections (E,F; 2 nM). The difference between the total (A,E) and non-specific (B, in the presence of 30 μM dipyridamole and F, in the presence of 100 μM dilazep) represents the specific binding of [^{3}H]NBMPR. Immunoreagents for von Willebrand Factor in conjunction with [^{3}H]biotin were used to produce autoradiograms of endothelial cells (C) or control (D, in the absence of primary antibody). Non-specific binding of avidin-[^{3}H]biotin is responsible for the high background (C,D). Rat sections that had been used for autoradiography were examined histologically and no evidence of destruction or removal of endothelial cells was found.


A
C
E
B
D
F

ventricular sections, indicating a homogeneous specific binding component (Fig. 3.2E,F). Histological examination of rat sections used for autoradiography indicated that endothelial cells were present and had not been destroyed during the experiments.

Von Willebrand Factor Radioimmunocytochemistry

Guinea pig cardiac sections incubated with primary antibody to von Willebrand Factor followed by secondary antibody and then avidin-[^{3}H]biotin complex gave rise to autoradiograms such as that depicted in Fig. 3.2C. Autoradiograms resulting from sections incubated in the secondary antibody and then in avidin-[^{3}H]biotin complex are represented by Fig. 3.2D. Autoradiograms were also produced from slides incubated only with avidin-[^{3}H]biotin (data not shown) and a high non-specific association of this complex was apparent. Further experiments were performed with avidin-peroxidase to investigate this high non-specific association. Similar results (data not shown) were obtained with and without secondary antibody incubations prior to avidin-peroxidase incubations. Therefore, the non-specific association of avidin to cardiac tissue caused the high non-specific binding of avidin-[^{3}H]biotin complex (Fig. 3.2D). The pattern produced by [^{3}H]biotin specific to the endothelial cell-located von Willebrand Factor (Fig. 3.2C) is similar to the high density [^{3}H]NBMPR binding component in these tissue sections (Fig. 3.2A).

Quantitative Autoradiography of [^{3}H]NBMPR Binding to Tissue Sections

Computer image analysis of the autoradiograms from guinea pig ventricular sections allowed the determination of NBMPR binding constants of the high and low binding site density components. K_D and B_{max} determinations were 1.4 nM and 1751 fmol/mg protein, respectively, for the high site density component and 4.5 nM and 990 fmol/mg protein, respectively, for the low site density component (Fig. 3.3).

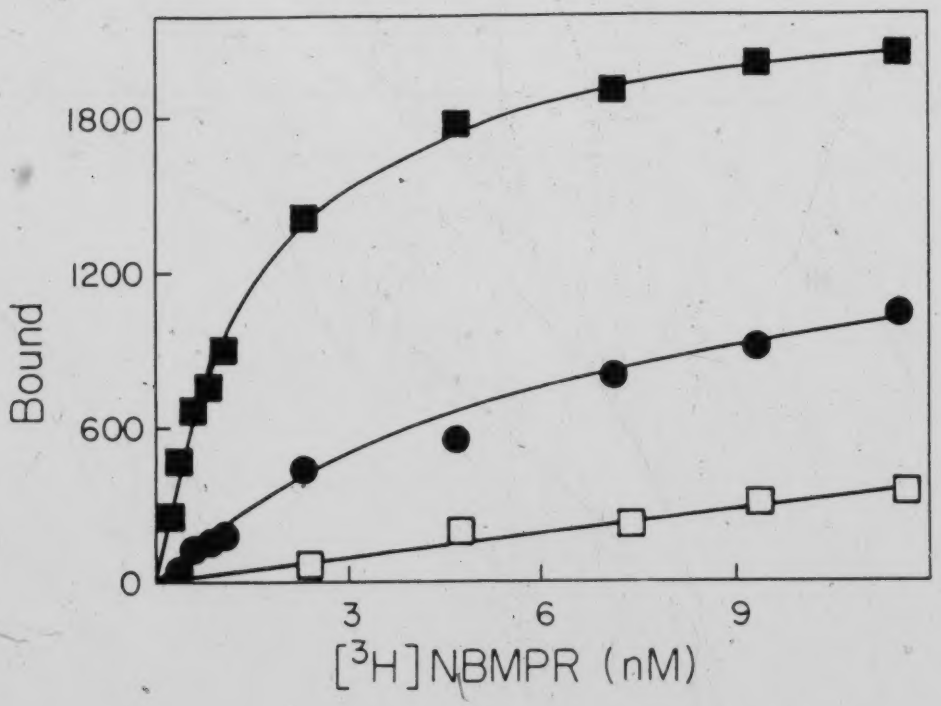
Similar analysis of rat ventricular sections, that possessed a uniform binding site distribution, provided NBMPR binding constants, K_D and B_{max}, of 0.6 nM and 526 fmol/mg protein, respectively (Fig. 3.4).

Dipyridamole inhibited NBMPR binding with K_i estimates of 108 nM and 245 nM for the high and low density binding components, respectively, of guinea pig ventricular sections and 3.1 μM for rat ventricular sections (Fig. 3.5). Inosine inhibited NBMPR binding to the high and low density components of guinea pig ventricular sections with K_i estimates of 179 μM and 238 μM, respectively (Fig. 3.6).

D. DISCUSSION

The distribution of NBMPR binding sites in cardiac sections was analyzed by autoradiography and a heterogeneous distribution in guinea pig and homogeneous distribution in

FIGURE 3.3: Concentration dependence of the binding of [^{3}H]NBMPR to guinea pig cardiac sections (upper panel). Computer image analysis allowed the separation of the high density areas (■ , total binding) associated with the vasculature, from the low density areas (● , total binding) representative of the cardiac myocytes. The non-specific binding (□) is linear with concentration. Abscissa: free [^{3}H]NBMPR concentration (nM). Ordinate: bound [^{3}H]NBMPR (fmol/mg protein). Points represent means of four sections from a single representative experiment. The non-specific line is a regression line through the points. Scatchard analysis (lower panel) of specific binding was used to determine [^{3}H]NBMPR binding constants for the two areas. Abscissa: site-specific bound [^{3}H]NBMPR (fmol/mg protein). Ordinate: site-specific bound [^{3}H]NBMPR (fmol/mg protein)/free [^{3}H]NBMPR concentration (nM). The lines are best-fit regression lines through the points; data correspond to the upper panel.


1800
1200
600
0
Bound
3
6
9
[3H]NBMPR (nM)

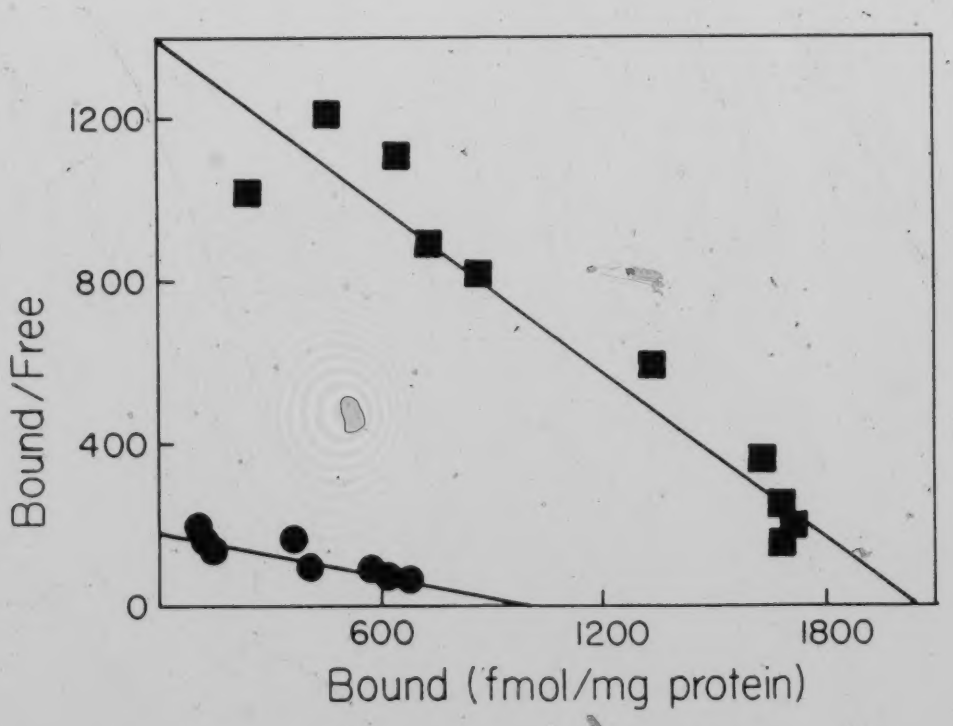
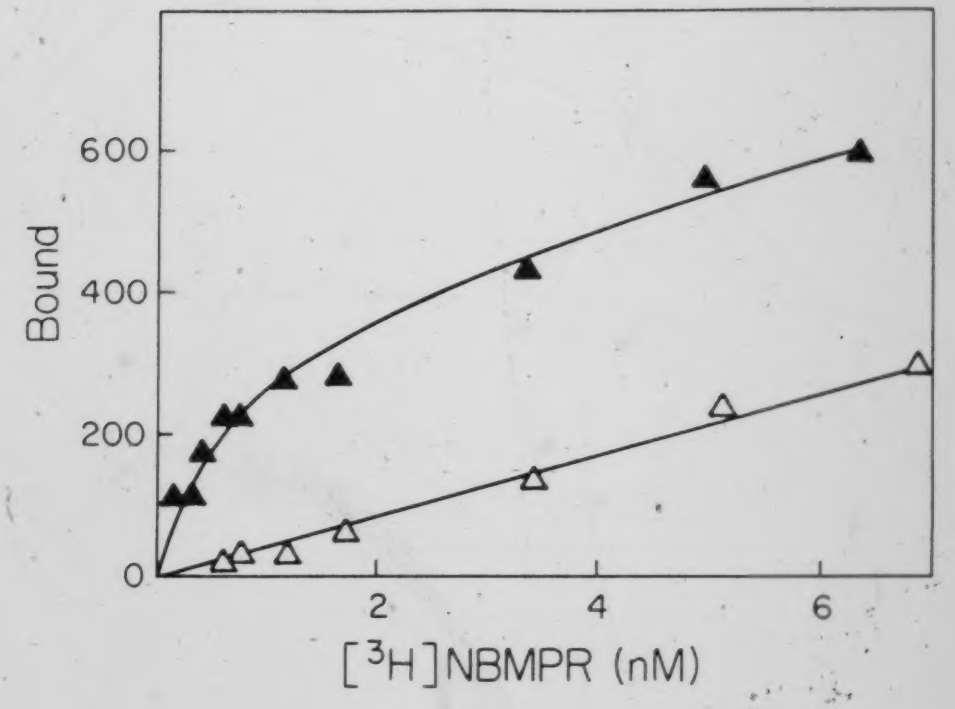

1200
800
400
0
Bound/Free
600
1200
1800
Bound (fmol/mg protein)

FIGURE 3.4: Concentration dependence of the binding of [^{3}H]NBMPR to rat cardiac sections (upper panel). Computer image analysis was used to measure total (▲) and non-specific (△) binding. Abscissa: free [^{3}H]NBMPR concentration (nM). Ordinate: bound [^{3}H]NBMPR (fmol/mg protein). Points represent means of four sections from a single representative experiment. The non-specific line is a regression line through the points. Scatchard analysis of specific binding (lower panel) was used to determine [^{3}H]NBMPR binding constants. Abscissa: site-specific bound [^{3}H]NBMPR (fmol/mg protein). Ordinate: site-specific bound [^{3}H]NBMPR (fmol/mg protein)/free [^{3}H]NBMPR concentration (nM). The line is best-fit regression line through the points; data correspond to upper panel. The scatter in the points around the line is due to the high affinity and low binding site density for [^{3}H]NBMPR in rat sections.


Bound
600
400
200
0
2
4
6
[3H]NBMPR (nM)

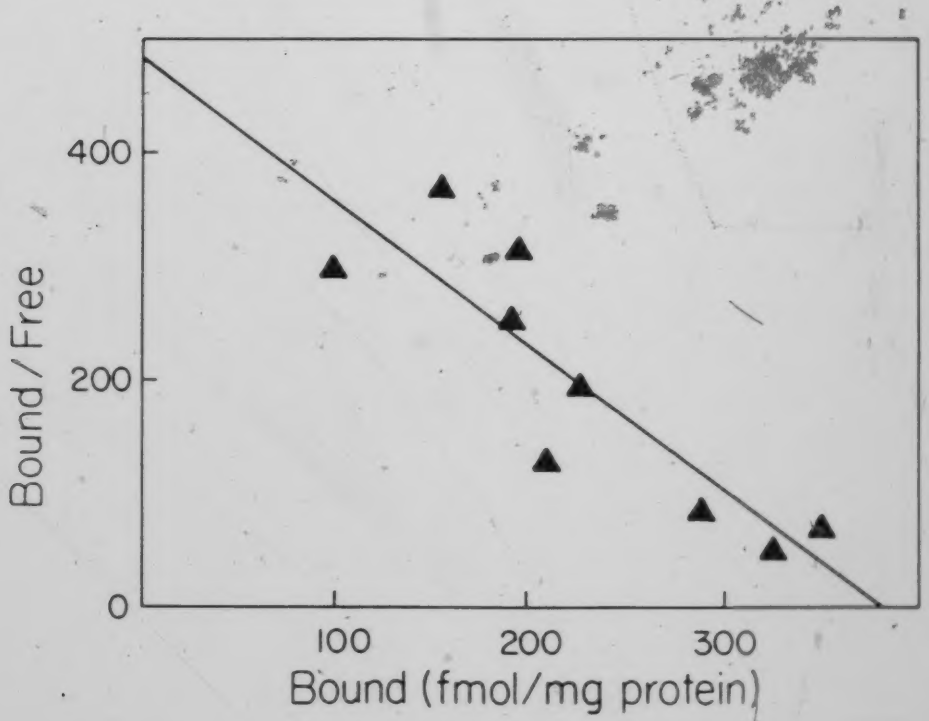
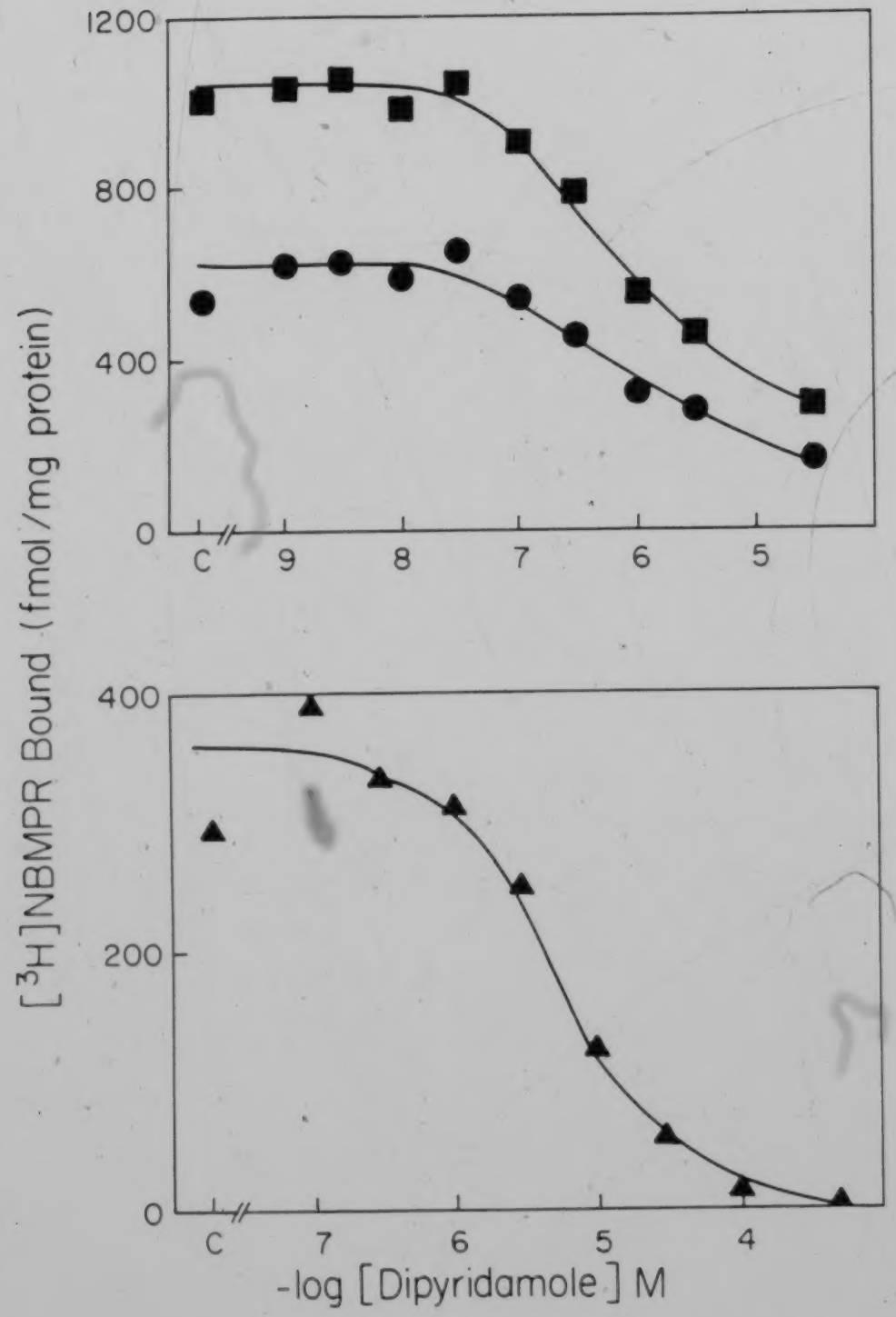

Bound/Free
400
200
0
100
200
300
Bound (fmol/mg protein)

FIGURE 3.5: Concentration-dependent inhibition of total [^{3}H]NBMPR binding by dipyridamole in guinea pig (upper panel; 3.7 nM [^{3}H]NBMPR) or rat (lower panel; 0.8 nM [^{3}H]NBMPR) cardiac sections. Computer image analysis allowed the separation of the high (■) and low (●) [^{3}H]NBMPR binding site density components found in guinea pig sections. Abscissas: $-\log_{10}$[dipyridamole] M. Ordinates: Total [^{3}H]NBMPR bound (fmol/mg protein). Points are means of four sections from single representative experiments.


1200
800
400
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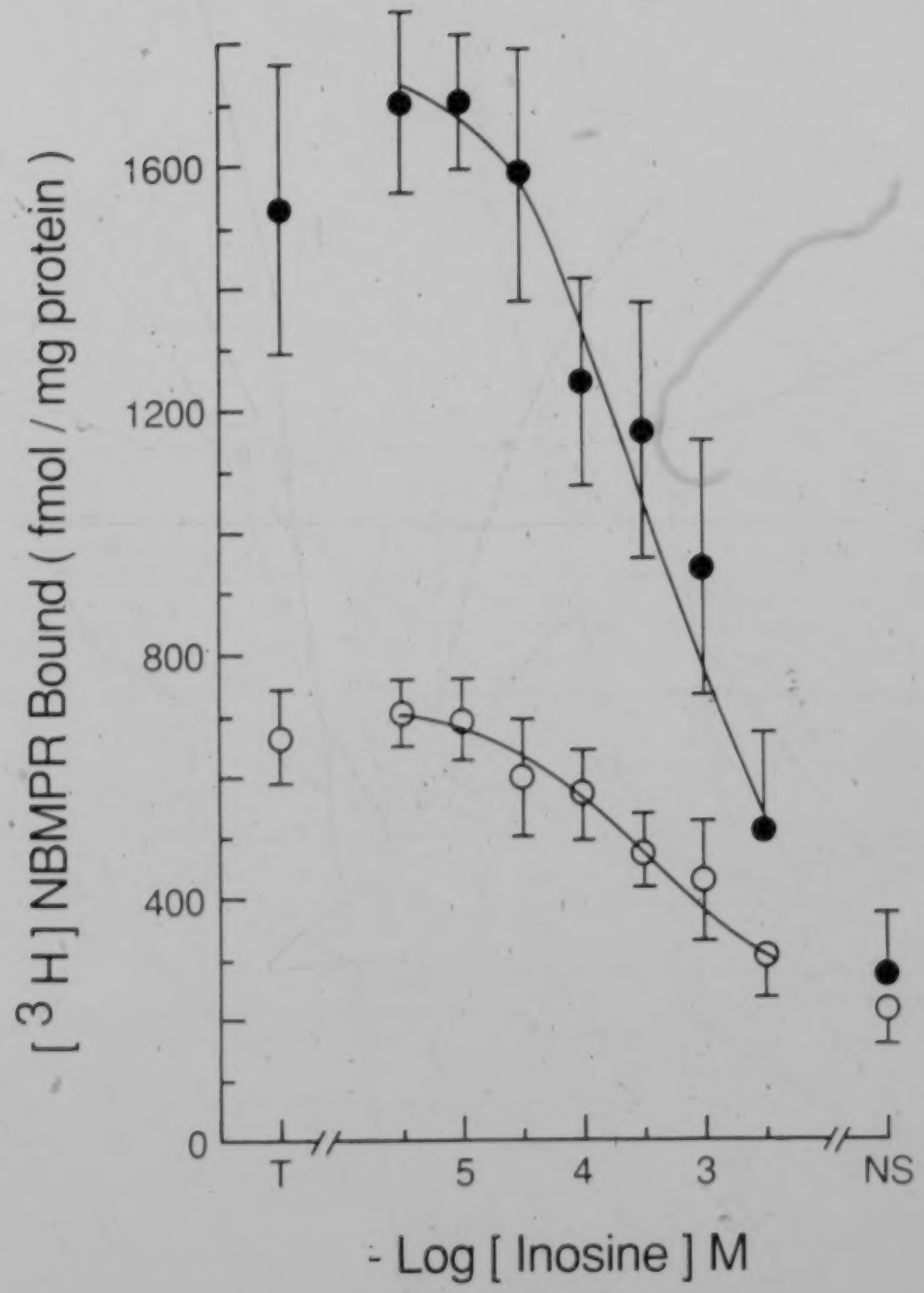
C
9
8
7
6
5
400
200
0
C
7
6
5
4
[3H]NBMPR Bound (fmol/mg protein)
-log [Dipyridamole] M

FIGURE 3.6: Concentration-dependent inhibition of total [^{3}H]NBMPR (3.2 nM) binding by inosine in guinea pig cardiac sections. Computer image analysis allowed the separation of the high (●) and low (○) [^{3}H]NBMPR binding site density components. Abscissa: $-\log_{10}$ [inosine] M. Ordinate: Total [^{3}H]NBMPR bound (fmol/mg protein). Points are means of eight sections from two animals; 95% confidence limits are shown. T, total binding in the absence of inhibitor; NS, non-specific binding determined by [^{3}H]NBMPR in the presence of 30 μM dipyridamole.


[3 H] NBMPR Bound (fmol / mg protein)
1600
1200
800
400
0
T
5
4
3
NS
- Log [Inosine] M

rat sections were found. Comparison of the high density NBMPR binding site distribution with that for von Willebrand Factor indicated endothelial cells in guinea pig heart have a high density of NBMPR sites.

Biochemical analysis of guinea pig sections resulted in a K_D value (3.2 nM) for NBMPR binding that was similar to values obtained for the high (1.4 nM) and low (4.5 nM) binding site areas analyzed by quantitative autoradiography. The B_{max} value obtained for the biochemical analysis (931 fmol/mg protein) was equivalent to that obtained for the low binding site density areas (990 fmol/mg protein) and approximately half that obtained for the high binding site density areas (1751 fmol/mg protein) analyzed by quantitative autoradiography. Since endothelial cells account for only 3% of heart volume (Nees et al., 1985), it is not unexpected that the B_{max} for the biochemical analysis approximates that for the low binding site density areas. Dipyridamole inhibition constants were similar when determined by biochemical analysis or quantitative autoradiography of the high and low binding site density areas. Both analytical techniques used 10 μm guinea pig ventricular sections, but while quantitative autoradiography separated the two binding areas, biochemical analysis provided values that were a composite of all cell types.

Inosine inhibition constants were similar for the high (179 μM) and low (238 μM) binding site density areas, as

determined by quantitative autoradiography. These K_i values for inhibition of NBMPR binding by inosine are typical of nucleoside transport systems of broad substrate specificity (Plagemann and Wohlhueter, 1980).

Analysis of [^{3}H]NBMPR binding to rat cardiac sections by the biochemical method and by quantitative autoradiography provided NBMPR binding constants that were similar: K_D values of 0.7 nM and 0.6 nM, respectively, and B_{max} values of 419 fmol/mg protein and 526 fmol/mg protein, respectively. Inhibition of NBMPR binding by dipyridamole also provided similar values for the two methods: K_i values of 3 μM by biochemical analysis and 3.1 μM by quantitative autoradiography. These results provide further evidence that rat and guinea pig cardiac tissue possess high affinity NBMPR sites that differ markedly in affinity for dipyridamole.

The species differences evident from this study are similar to those reported for cardiac membranes (Williams et al., 1984). Isolated cardiac myocytes from these species have also been investigated (Clanachan et al., 1987) and the maximum NBMPR binding site density was two-fold greater in guinea pig myocytes than in rat myocytes. This corresponds to the differences between the low binding site density areas in guinea pig cardiac sections and rat cardiac sections reported in this study. This supports the interpretation that the low binding site density areas in guinea pig sections are representative of cardiac myocytes.

A comparison of the distributions of von Willebrand Factor and the high density NBMPR binding component in consecutive sections of guinea pig ventricles indicated co-localization of these markers. Von Willebrand Factor, a component of clotting Factor VIII required for platelet aggregation and attachment, is synthesized and released by endothelial cells and thus serves as a specific marker for endothelial cells (Jaffe, 1984; McComb et al., 1982). In heart tissue, von Willebrand Factor has been localized in surface endocardial cells and coronary vascular endothelium (McComb, 1984). The co-localization of von Willebrand Factor and the high density NBMPR binding component found in this study indicates that guinea pig ventricular endothelial cells have a high density of NBMPR binding sites and nucleoside transport sites.

Significant endothelial cell uptake of adenosine has been described in canine skeletal muscle (Gorman et al., 1986), guinea pig heart (Nees et al., 1985; Sparks et al., 1985), cultured guinea pig coronary endothelium (Nees et al., 1985) and cultured pig aortic (Pearson et al., 1978; 1983) and pulmonary (Pearson and Gordon, 1985) endothelial cells. However, those studies did not distinguish between transport of adenosine into cells and subsequent metabolism. From our studies, it would appear that guinea pig coronary endothelial cells have a high capacity for nucleoside transport due to the high density NBMPR binding sites. However, without

kinetic data on membrane fluxes of nucleosides in these cells, their true capacity for nucleoside transport is not known.

Nucleoside transport systems differ among species and tissues; facilitated diffusion systems have usually been subclassified on the basis of inhibitor sensitivity. Rat tissues have been shown to be less sensitive to dipyridamole than tissues from guinea pig and other species, when potentiation of adenosine effects (Hopkins and Goldie, 1971; Williams et al., 1984), inhibition of nucleoside transport or inhibition of NBMPR binding is measured (Hammond and Clanachan, 1985; Clanachan et al., 1987). Additionally, some CNS membrane preparations with apparently uniform populations of NBMPR binding sites show biphasic dipyridamole inhibition of NBMPR binding (Hammond and Clanachan, 1984; 1985). This suggests two or more forms of the transporter that can be distinguished by dipyridamole, but not by NBMPR. From this study, it was apparent that the high and low density NBMPR binding components in guinea pig heart were sensitive to dipyridamole.

While it has been clearly shown that NBMPR is a high affinity, nucleoside transport inhibitor in rat and guinea pig cardiac myocytes (Heaton and Clanachan, 1987; Clanachan et al., 1987; Ford and Rovetto, 1987), it is possible that rat endothelial cells may have an NBMPR-insensitive transport component akin to transport systems of some cultured cells (Paterson et al., 1987). For instance, rat erythrocytes

possess both NBMPR-sensitive and -insensitive transport components (Jarvis, 1987) and these two systems may be co-localized on other cell types.

According to the adenosine hypothesis, adenosine is produced and released from cardiac cells during times of metabolic stress. It acts locally to produce many receptor-mediated effects that reduce excitability and contractility, and increase oxygen supply (Berne, 1980; Dobson and Fenton, 1983). Subtypes of nucleoside transport systems among various regions of heart could have differing drug affinities, thus allowing selective potentiation of some adenosine-mediated effects. Alternatively, regional differences in nucleoside transporter density could be important in controlling the rate of removal of adenosine from its sites of action. Nucleoside transport inhibitors would be predicted to exert greater potentiation of adenosine in areas of higher transporter density. Therefore, regional differences in nucleoside transport systems, with respect to drug affinity or site density, could have important therapeutic implications.

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CHAPTER 4

SUBTYPES OF NUCLEOSIDE TRANSPORT INHIBITORY SITES IN HEART: A QUANTITATIVE AUTORADIOGRAPHICAL ANALYSIS[4]

A. INTRODUCTION

Adenosine is an endogenous nucleoside that exerts important regulatory effects on cardiovascular function. Stimulation of A_1 adenosine receptors produces negative inotropic, chronotropic and dromotropic effects while activation of A_2 adenosine receptors causes vascular smooth muscle relaxation.

Adenosine is cleared from the vicinity of its cell surface receptors by rapid uptake systems consisting of membrane-located nucleoside transport mechanisms and subsequent metabolism. Many of the coronary vasodilators, for example dipyridamole and dilazep, exert some of their therapeutic effects by interacting with transport inhibitory sites associated with the nucleoside transport mechanism. In this way, adenosine influx is inhibited and its levels close to adenosine receptor sites are sustained, leading to potentiation and prolongation of adenosine-mediated effects (Williams et al., 1984).

Many studies of drug interactions with nucleoside transport mechanisms have utilized the high affinity probe

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[4] A version of this chapter has been published. Parkinson, F.E. and Clanachan, A.S. 1989. Eur. J. Pharmacol. 163:69-75.

[^{3}H]nitrobenzylthioinosine ([^{3}H]NBMPR) (Cass et al., 1974). NBMPR binds with high affinity and selectivity to membrane-located sites in many cell types including erythrocytes and cardiac myocytes. Binding to these sites inhibits nucleoside transport and inhibition constants of drugs for the inhibition of [^{3}H]NBMPR binding is predictive of their transport inhibitory activity (Hammond et al., 1983; Clanachan et al., 1987).

Subtypes of facilitated diffusion nucleoside transport systems have recently been identified. Systems of high or low sensitivity to NBMPR are present in several lines of cultured cells (Paterson et al., 1987) and systems of high and low sensitivity to dipyridamole are detectable in guinea pig and rat myocytes, respectively (Clanachan et al., 1987). However, it is not known if such subtypes are present in the membranes from a single cardiac cell, or if they are present on different cell types within heart from a single species. The discovery of regional subtypes differing in drug affinity might allow the selective pharmacological modulation of the regulatory actions of adenosine.

In a previous autoradiographical study (Parkinson and Clanachan, 1989) we have shown that the distribution of [^{3}H]NBMPR binding sites in guinea pig hearts is non-uniform. A high binding site density is associated with the vascular endothelium, as defined by co-localization with von Willebrand Factor at the light microscopic level. A lower

binding site density is evident over cardiac myocytes. In the present study, we have examined for other differences between transporters of these two areas. Using quantitative autoradiography, we have determined inhibition constants for several nucleoside transport inhibitors and substrates for inhibition of [^{3}H]NBMPR binding in these different areas of guinea pig heart.

B. METHODS

Tissue Preparation

Hartley guinea pigs (males, 200-400 g) were killed by a blow to the head followed by exsanguination. The hearts were quickly removed and the ventricles were embedded in sectioning media (20 parts OCT Compound:10 parts distilled water: 7 parts tragacanth gum), frozen in isopentane cooled with liquid nitrogen, and stored at -70^{o}C for up to two weeks. Transverse sections (10 μm) were cut (-22^{o}C) with a cryostat (International Equipment Company model CT1), mounted onto gelatin-coated slides, desiccated overnight at 4^{o}C and then used immediately or stored at -70^{o}C for up to two weeks.

Drug Competition with [^{3}H]NBMPR for Binding to Tissue Sections

Two microscope slides with two cardiac sections per slide were used for each determination. The sections were preincubated for 5 min in ice-cold phosphate-buffered saline (PBS; NaCl 137 mM, Na_2HPO_4 6mM, KCl 2.7 mM, KH_2PO_4 1.5 mM,

$CaCl_2$ 0.9 mM, $MgCl_2$ 0.5 mM; pH 7.4), then incubated with [^{3}H]NBMPR (2.5-4.0 nM) in the presence of graded concentrations of competitor for 30 min at 20°C. Fixation of these tissue sections resulted from the addition of formalin to the incubation medium (final conc. 0.4%) for 10 min. This method of fixation was found not to alter the total or non-specific binding of [^{3}H]NBMPR, as determined by using scintillation spectrometry to measure the tritium associated with fixed and unfixed sections scraped from slides. The tissue sections were washed twice for 5 min in ice-cold PBS then rinsed three times in ice-cold distilled water. Total binding was determined in the absence of competitor; non-specific binding was determined in the presence of 30 μM dipyridamole. Tissue sections were blown dry with cold air in a cold room (4°C) and desiccated overnight. The tissue sections were apposed to Ultrofilm ^{3}H in x-ray cassettes and stored in the dark at 4°C for three weeks. Included with each film were two sets of six tritium standards (Parkinson and Clanachan, 1989). The films were developed using Microdol X Developer for 10 min, rinsed with distilled water, fixed with Kodak Fixer for 5 min, and washed for 20 min, all at 20°C.

Quantitation of Autoradiograms

A computer image analysis system (Imageplus, Scientific Microprograms) was used to determine optical densities of different areas of the autoradiograms: the high

(endothelial) and low (myocyte) [^{3}H]NBMPR binding site density components in guinea pig cardiac sections (Fig. 4.1). Each experimental condition included a minimum of eight tissue sections. Autoradiograms were coded and analyzed in random order by an individual who was not aware of the experimental condition.

In order to convert the computer-derived optical density values to fmol NBMPR bound/mg protein, a standard curve (ln dpm/mg dry weight versus ln optical density) was prepared for each sheet of Ultrofilm ^{3}H used (Unnerstall et al., 1982). Experimental optical density values were converted to dpm/mg dry weight using the standard curves, to fmol NBMPR bound/mg dry weight using the specific activity of the ligand, and to fmol NBMPR bound/mg protein using the conversion factor of 1.4 mg dry weight/mg protein. This factor was determined from Lowry protein assays (Lowry et al., 1951) on dried samples of guinea pig heart solubilized in 1N NaOH.

Calculation of Inhibition Constants

Linear interpolation from the inhibition curves at the 50% mark was used to determine IC_{50} values for the endothelial and myocyte binding site components for each experiment. Inhibition constants were determined using the Cheng and Prusoff (1973) equation: $K_i = IC_{50}/(1 + [L]/K_D)$ where [L] is the concentration of [^{3}H]NBMPR used and K_D is 1.4 or 4.5 nM for the high and low site density areas, respectively, as determined by quantitative autoradiographical analysis

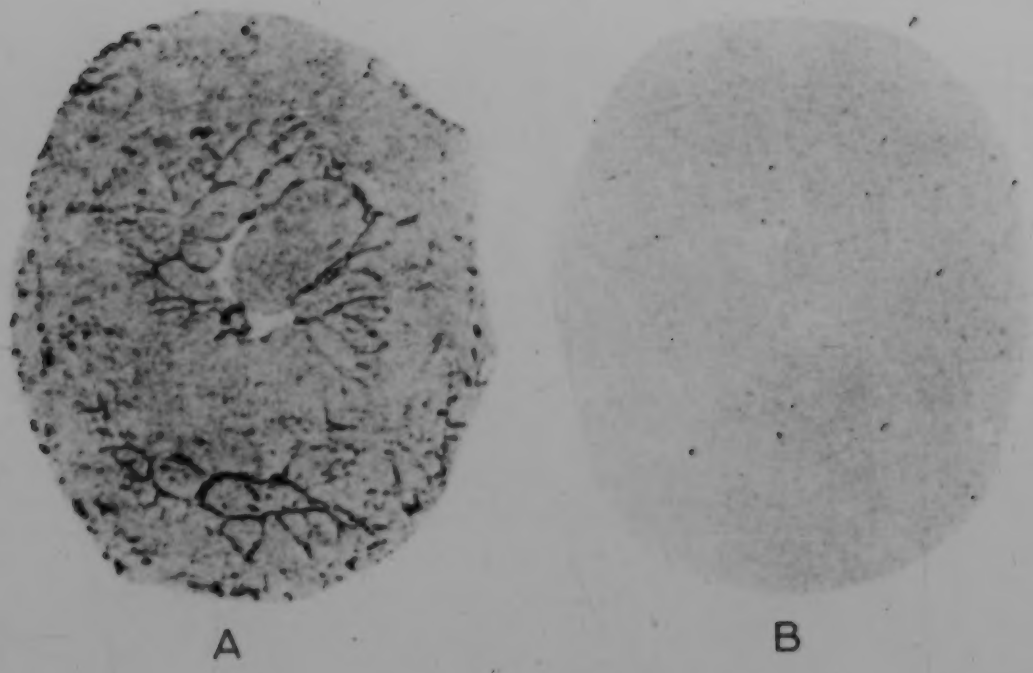

FIGURE 4.1; [^{3}H]NBMPR binding autoradiograms. The difference between total (A, 8 nM [^{3}H]NBMPR) and non-specific (B, 8 nM [^{3}H]NBMPR in the presence of 30 μM dipyridamole) binding is the specific binding component, which has a heterogeneous distribution. The high site density areas correspond to coronory endothelial cells and the low site density areas correspond to cardiac myocytes.

of saturation experiments (Parkinson and Clanachan, 1989). Geometric means of the K_i values were calculated for each competing ligand for the two binding site areas.

Materials

Drugs and radioligand for these studies were obtained from the following sources: [G-^{3}H]nitrobenzylthioinosine (sp. act. 37 Ci/mmol), from Moravek Biochemicals, CA, and repurified as necessary before use; dipyridamole, nitrobenzylthioguanosine, adenosine, inosine, and uridine, from Sigma Chemical Co., St. Louis, MO; Ultrofilm ^{3}H, from Fisher Scientific, Edmonton, Alta.; OCT Compound, from Canlab, Edmonton, Alta. Other compounds, donated by their manufacturers, were as follows: dilazep (Hoffman-La Roche Ltd., Quebec); soluflazine, mioflazine and lidoflazine (Janssen Pharmaceutica, Belgium); and hexobendine (Chemie Linz AG, Austria). Nitrobenzylthioinosine was kindly provided by Dr. A.R.P. Paterson (McEachern Laboratory), University of Alberta.

C. RESULTS

Quantitative Autoradiography of Competition Experiments

Computer image analysis of the autoradiograms allowed the determination of [^{3}H]NBMPR bound, to endothelial cells and to myocytes, in the presence of graded concentrations of the following competitors: NBMPR (10^{-11}-$3x10^{-7}$ M),

nitrobenzylthioguanosine (NBTGR; 10^{-10}-$3x10^{-7}$ M), dipyridamole (10^{-9}-$3x10^{-5}$ M), dilazep (10^{-10}-$3x10^{-6}$ M), hexobendine (10^{-9}-$3x10^{-5}$ M), soluflazine ($3x10^{-9}$-$3x10^{-5}$ M), mioflazine (10^{-9}-$3x10^{-5}$ M), lidoflazine (10^{-9}-$3x10^{-5}$ M), adenosine ($3x10^{-6}$-$3x10^{-3}$ M), inosine ($3x10^{-6}$-$3x10^{-3}$ M), and uridine ($3x10^{-6}$-$3x10^{-3}$ M). The concentrations of [^{3}H]NBMPR used in these experiments were greater than the K_D for the endothelial cells and less than the K_D for the myocytes. Thus the total binding was 2-3 fold higher in the endothelial cell areas (Fig. 4.2) than in myocyte areas. Dipyridamole, used to determine non-specific binding, reduced [^{3}H]NBMPR to similar binding levels in the two areas. Of the binding inhibitors used in the experiments of Fig. 4.2, NBTGR had highest affinity for both binding areas while inosine had lowest affinity.

In order to identify more readily differences in affinity of each drug for the two cell types, site-bound [^{3}H]NBMPR was expressed as a percentage of total minus non-specific binding (Fig. 4.3). Inosine and NBTGR showed little selectivity, but lidoflazine had higher affinity for the endothelial cell areas than for the myocyte areas.

A summary of the inhibition constants and potency ratios (K_i for myocytes divided by K_i for endothelial cells) for all of the compounds tested is given in Table 4.1. NBMPR and its congener NBTGR had highest affinities for both areas while the nucleoside transporter substrates, adenosine, inosine, and

FIGURE 4.2: Concentration-dependent inhibition of total [^{3}H]NBMPR binding by NBTGR (upper panel), lidoflazine (center panel), and inosine (lower panel) in guinea pig heart. Computer image analysis allowed the separation of endothelial (●) and myocyte (○) [^{3}H]NBMPR binding components. Abscissae: $-\log_{10}$[inhibitor] M. Ordinates: total [^{3}H]NBMPR bound (fmol/mg protein). Points are means of at least eight sections from two or more animals; 95% confidence limits are shown unless they are covered by the symbols. T, total binding in the absence of inhibitor; NS, non-specific binding determined by [^{3}H]NBMPR in the presence of 30 μM dipyridamole; [^{3}H]NBMPR concentration was 3.2-4.0 nM.

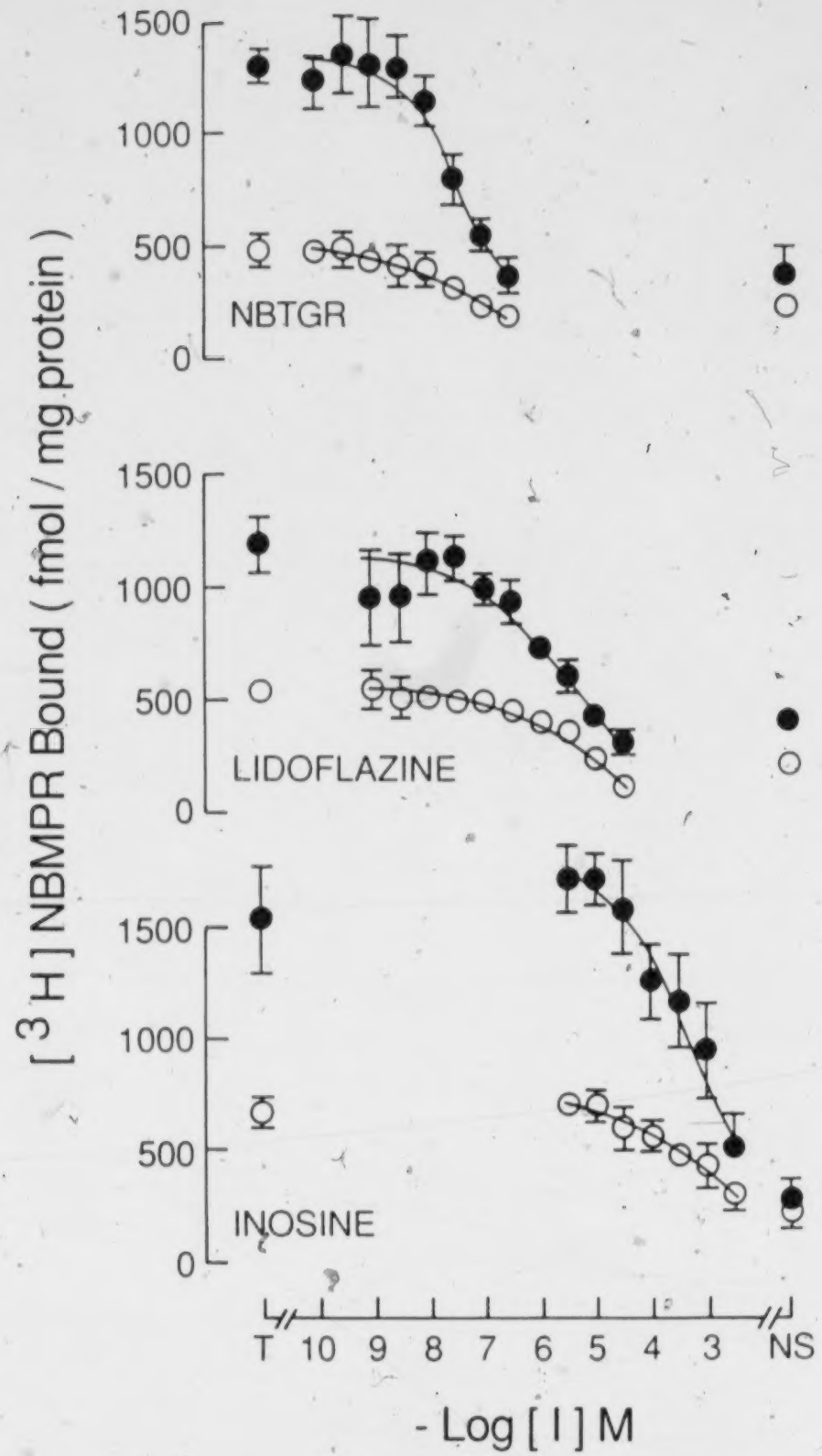
1500
1000
500
0
NBTGR
LIDOFLAZINE
INOSINE
[3 H] NBMPR Bound (fmol / mg.protein)
T 10 9 8 7 6 5 4 3 NS
- Log [I] M

FIGURE 4.3: Concentration-dependent inhibition of specific [^{3}H]NBMPR binding by NBTGR (upper panel), lidoflazine (center panel), and inosine (lower panel) in guinea pig heart. Computer image analysis allowed the separation of the endothelial (●) and myocyte (○) [^{3}H]NBMPR binding components. Abscissae: $-\log_{10}$[inhibitor] M. Ordinates: % specific [^{3}H]NBMPR bound. Data are means of four sections from single representative experiments. T, 100%, specific binding in the absence of inhibitor; NS, 0%, non-specific binding determined by [^{3}H]NBMPR in the presence of 30 μM dipyridamole; [^{3}H]NBMPR concentration was 3.3 nM, 3.2 nM, and 3.3 nM for NBTGR, lidoflazine, and inosine, respectively.

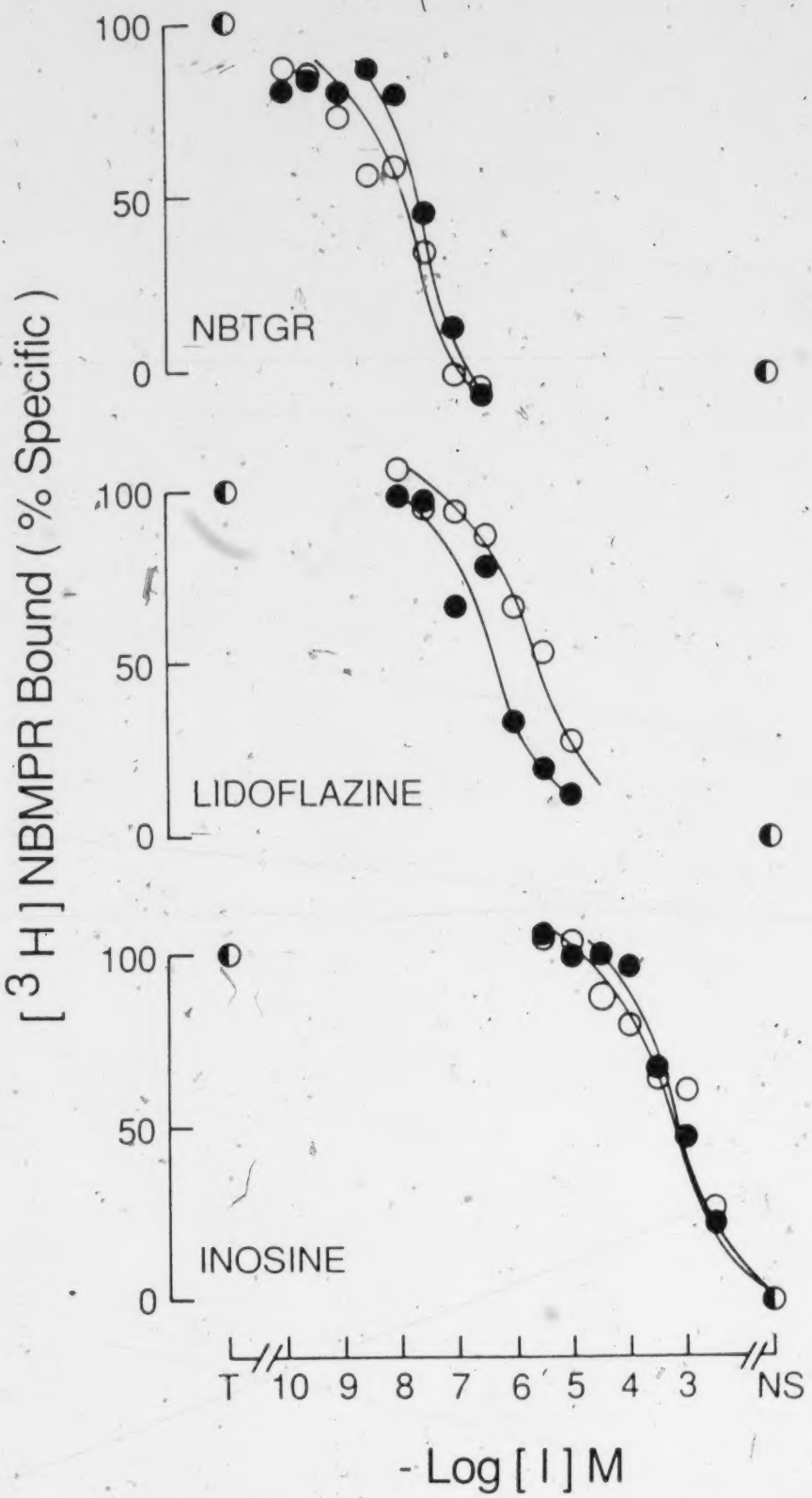
100
50
0
NBTGR
LIDOFLAZINE
INOSINE
[3 H] NBMPR Bound (% Specific)
T 10 9 8 7 6 5 4 3 NS
- Log [I] M

TABLE 4.1: Quantitative autoradiographical analysis of inhibition of [^{3}H]NBMPR binding from endothelial cells and cardiac myocytes in guinea pig cardiac sections by nucleoside transporter inhibitors and substrates. Inhibition constants were calculated from the formula $K_i = IC_{50}/(1+[L]/K_D)$; [L] is the [^{3}H]NBMPR concentration used and K_D is 1.4 or 4.5 nM for the endothelial and myocyte areas, respectively. Seven to ten concentrations of each inhibitor plus total and non-specific conditions were used to define inhibition curves. A minimum of eight tissue sections from two or more animals was used for each experimental condition. In most instances, the standard deviations for the determinations of site-bound [^{3}H]NBMPR were less than 20%.

Inhibitor	K_i (Inhibition Constants, nM) Endothelium	Myocytes	Potency Ratio $\frac{K_i \text{ Myocytes}}{K_i \text{ Endothelium}}$
NBMPR	2	6	3.0
NBTGR	13	16	1.2
Dipyridamole	108	245	2.3
Dilazep	21	55	2.6
Hexobendine	92	584	6.3
Soluflazine	31	74	2.4
Mioflazine	77	226	2.9
Lidoflazine	191	1,570	8.2
Adehosine	73,000	136,000	1.9
Inosine	179,000	238,000	1.3
Uridine	647,000	1090,000	1.7

uridine, had the lowest affinities for [^{3}H]NBMPR binding sites in these areas. While most of the compounds showed 3-fold or less selectivity, similar to that seen with [^{3}H]NBMPR, lidoflazine had 8.2-fold and hexobendine had 6.3-fold selectivity for the endothelial cell binding component.

D. DISCUSSION

In our previous study (Parkinson and Clanachan, 1989) we showed that the distribution of [^{3}H]NBMPR binding sites in guinea pig heart is heterogenous with the high site density areas corresponding to coronary vessel endothelium and the low site density areas corresponding to cardiac myocytes. This provided us with a model in which to determine the affinity of known nucleoside transport inhibitors and substrates for transporter sites on different cell types within a single tissue.

Nucleoside transport systems are known to differ among species and tissues in many important criteria. Facilitated diffusion systems have usually been subclassified on the basis of sensitivity to inhibitors, primarily dipyridamole and NBMPR. It has long been recognized that dipyridamole is unable to potentiate the effects of adenosine in rat tissue (Hopkins and Goldie, 1971). More recently it has been shown that dipyridamole, hexobendine and lidoflazine have significantly lower affinity for nucleoside transport systems in rat tissues compared with other species, particularly

guinea pig. This is true either when inhibition of NBMPR binding or potentiation of adenosine action is measured (Williams et al., 1984). Additionally, some CNS membrane preparations with apparently uniform populations of NBMPR binding sites were inhibited by dipyridamole and dilazep in a biphasic manner (Hammond and Clanachan, 1984; 1985). This suggests two or more forms of the transporter which can be distinguished by some transport inhibitors, but not by NBMPR. The results of the present study indicate that dipyridamole and dilazep have little capacity to distinguish the two NBMPR binding site components in guinea pig heart. However, of all the compounds tested, lidoflazine and hexobendine have the greatest potential to inhibit selectively nucleoside transport in the endothelial cell component.

NBMPR sensitivity has also been used as a criterion to classify nucleoside transporters. NBMPR-insensitive transporters have been reported on several cultured cells, mainly from tumor cells derived from rat and mouse tissues, and rat erythrocytes (Paterson et al., 1987; Jarvis, 1987). It has clearly been shown, however, that NBMPR is a high affinity nucleoside transport inhibitor in guinea pig cardiac myocytes (Heaton and Clanachan, 1987). Unfortunately, nucleoside transport systems have not been studied directly in freshly isolated or cultured endothelial cells. Instead, for many species, nucleoside uptake (transport followed by intracellular metabolism and/or efflux) into endothelial cells

was measured and found to be sensitive to NBMPR or dipyridamole (Pearson et al., 1978; Pearson and Gordon, 1985; Gorman et al., 1986). The affinity of [^{3}H]NBMPR, determined by quantitative autoradiographical analysis of saturation experiments, was 3-fold greater in the endothelial cell binding component than in cardiac myocytes (Parkinson and Clanachan, 1989).

Another study that suggested subtypes of transporters between cell types was that of Van Belle et al. (1986). They presented data from dogs that suggested mioflazine did not inhibit adenosine efflux from cardiac myocytes but did inhibit adenosine influx and inosine efflux from coronary endothelium. Based on this report, we used mioflazine and an analogue, soluflazine, to inhibit the two components of [^{3}H]NBMPR binding in guinea pig cardiac sections, but no marked differences in affinity were found between the two binding site areas. These compounds had high affinities for these sites with K_i values similar to dipyridamole and dilazep.

Geiger and co-workers (Geiger and Nagy, 1984; Geiger et al., 1985) have attempted to distinguish nucleoside transport systems on the basis of substrate specificity for adenosine. They hypothesized that neuronal systems are selective for adenosine (K_m = 1-10 μM) over other nucleosides while peripheral systems have lower affinity for adenosine (K_m = 100-1000 μM) and broad specificity. We felt it of interest

to test whether these two systems might be associated with different cell types present within one tissue, but adenosine, inosine and uridine had little selectivity for NBMPR binding site areas in guinea pig cardiac sections. The K_i values for adenosine and the similar affinities of the three substrates indicate both areas are typical for peripheral nucleoside transport systems.

Many attempts have been made to classify nucleoside transport systems on the basis of inhibitor or substrate selectivity. This is the first study to examine for these subtypes in the different cell types of cardiac tissue. From our results it would appear that lidoflazine and hexobendine have the greatest potential to inhibit selectively the nucleoside transporter associated with coronary endothelial cells. Considering the diverse cardiovascular effects of adenosine, the ability to potentiate some of these effects in defined regions of the heart through the use of transport inhibitors could be of therapeutic importance.

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CHAPTER 5

AUTORADIOGRAPHY OF ADENOSINE RECEPTORS AND NUCLEOSIDE TRANSPORT SITES IN CARDIAC CONDUCTION CELLS

A. INTRODUCTION

Adenosine is an important modulator of cardiovascular function. As early as 1929, it was observed that adenosine has negative chronotropic and dromotropic effects in heart; of the species studied, the effect on the sinoatrial node was predominant in dog, cat and rabbit while the atrioventricular node effect was predominant in guinea pig (Drury and Szent-Gyorgyi, 1929). In man, both sinus rate and atrioventricular conduction are depressed by intravenous adenosine administration and this is of therapeutic benefit in some forms of paroxysmal supraventricular tachycardia (DiMarco et al., 1983). Additionally, it has been proposed that conduction delays, seen in cases of ischemia and hypoxia, may be due to adenosine released from cardiac cells under hypoxic stress (Belardinelli et al., 1980; West and Belardinelli, 1985b; Clemo and Belardinelli, 1986a; 1986b).

Several reports by Belardinelli and colleagues have described the electrophysiological effects of adenosine and adenine nucleotides in preparations containing conduction cells (Belardinelli et al., 1980; 1982; 1984; West and Belardinelli, 1985a; 1985b; Clemo and Belardinelli, 1986a; 1986b). These effects of adenosine are mediated by cell surface receptors of the A_1 subtype (Clemo and Belardinelli,

1986a) and stimulation of these receptors increases potassium conductance and produces hyperpolarization (Sperelakis, 1987). Nucleoside transport inhibitors, such as dipyridamole and nitrobenzylthioinosine (NBMPR), potentiate whereas the adenosine receptor antagonist aminophylline inhibits the cardiovascular actions of adenosine (Belardinelli et al., 1982; 1984).

Adenosine A_1 receptors are present in low density in cardiac tissue. Estimates of the maximum site density are about 15 and 30.5 fmol/mg protein in rat (Linden et al., 1985; Martens et al., 1987) and bovine (Lohse et al., 1985) crude ventricular membrane preparations, respectively. A more purified preparation from pig atria provided an estimate of maximum receptor density of 32 fmol/mg protein (Leid et al., 1988). The density of A_1 receptors has not been determined in cells of the impulse conduction system; however it is possible that increased receptor density on these cells may account for the predominant chronotropic and dromotropic effects of adenosine in heart.

Another possibility is that altered adenosine metabolism, for example, decreased transport into conduction cells, may allow increased adenosine-mediated effects in these cells. In support of this idea, we have shown in previous studies that nucleoside transport sites identified with [^{3}H]NBMPR have a greater site density in coronary endothelial cells than in ventricular cardiac myocytes (Parkinson and Clanachan, 1989a;b). Other cell types within

heart may also exhibit differences in transporter density.

Other factors such as increased receptor affinity for adenosine or increased coupling efficiency between receptors and their transduction mechanisms could also account for the enhanced effectiveness of adenosine on conduction cell function relative to other cardiac myocytes.

In this study, we examined whether guinea pig atrioventricular conduction cells had altered receptor or nucleoside transporter densities relative to other cardiac myocytes. At present, it is not possible to purify conduction cells, separate from the other cellular components of heart. Therefore these cells were identified histologically and autoradiography was performed with the A_1 receptor antagonist 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine (DPCPX) and the nucleoside transport inhibitor [^{3}H]NBMPR. In this way, site densities of receptors and transport inhibitory sites in conduction cells relative to other cardiac myocytes were determined.

B. METHODS

Guinea pig hearts were rapidly removed, and from each heart the area of right atrium containing coronary sinus and inferior vena cava, plus the interatrial septum and the upper part of the interventricular septum was isolated. This preparation contains atrioventricular conduction cells (Clemo and Belardinelli, 1986a). The tissues were embedded in sectioning media (20 parts OCT Compound: 10 parts distilled

water: 7 parts tragacanth gum), frozen in isopentane cooled with liquid nitrogen and stored at -70°C for up to two weeks. Sections (10 μm) were cut (-22°C) parallel to the long axis of the heart with a cryostat (International Equipment Company model CT1), mounted onto gelatin-coated slides, desiccated overnight at 4°C and then used immediately or stored at -70°C for up to two weeks.

Acetylcholinesterase Histochemistry

Cardiac conduction cells can be reliably identified by positive staining for acetylcholinesterase (AChE) (Anderson, 1972; Bojsen-Møller and Tranum-Jensen, 1971). Vagal innervation of cardiac tissue is concentrated on impulse conducting cells within the atria, and both the nerves and the innervated cells are characterized by having large quantities of AChE. The procedure used for detecting AChE in these cells was that described by El-Badawi and Schenk (1967).

Von Willebrand Factor Immunohistochemistry

Von Willebrand Factor is a component of clotting factor VIII and is a reliable marker for endothelial cells. An immunocytochemical method was used to localize these cells. Sections that had been frozen were first preincubated for 5 min in ice-cold PBS (NaCl 137mM, Na_2HPO_4 6mM, KCl 2.7mM, KH_2PO_4 1.5mM, $CaCl_2$ 0.9mM, $MgCl_2$ 0.5mM; pH 7.4). This was followed by 10 min in 0.4% formaldehyde in ice-cold PBS and two subsequent 5 min washes in ice-cold PBS. Sections were then incubated in presoak buffer (5% bovine serum albumin

(BSA), 0.05% NaN_3, 0.3% Triton X100 in PBS) for one hour at 20°C. Washes, at this and later steps, were performed with 1% BSA and 0.05% NaN_3 in PBS, 2 x 5 min at 20°C. Sections were then incubated with the primary antibody, rabbit polyclonal antibody to human von Willebrand Factor (1:1600 dilution in 1% BSA, 0.05% NaN_3, 0.3% Triton X100 in PBS), for 1 hr at 20°C. Sections were washed then incubated with the secondary antibody, biotinylated goat anti-rabbit IgG (1:400 dilution in the same buffer used for primary antibody), for 1 hr at 20°C. Sections were washed again then incubated with horseradish peroxidase avidin D (1:100 dilution in the same buffer used for washes), for 1 hr at 20°C. Sections were washed again then incubated with 0.05% diaminobenzidine and 0.001% H_2O_2 in PBS, for 5 min at 20°C in the dark. Sections were washed again, then dehydrated and mounted.

[^{3}H]NBMPR Binding

Sections were preincubated in ice-cold PBS for 5 min, then incubated in 10 nM [^{3}H]NBMPR for 30 min at 20°C. Fixation of the tissue sections (10 min) resulted from the addition of neutral buffered formaldehyde (final conc. 0.4%) to the incubation medium for 10 min. Sections were washed twice for 5 min with ice-cold PBS and rinsed three times with ice-cold distilled water. Sections were blown dry with cold air and desiccated overnight. The amount of [^{3}H]NBMPR that bound in the presence of 30 μM dipyridamole was termed the non-specific binding component.

[^{3}H]DPCPX Binding

Sections were preincubated in PBS, 4°C for 5 min, then incubated in 5 nM [^{3}H]DPCPX for 2 hr, 20°C. The results of preliminary experiments indicated that [^{3}H]DPCPX bound to guinea pig cardiac membranes with a K_D value of 1.5 nM (0.6-3.9). Tissue sections were then processed as described for [^{3}H]NBMPR binding assays. Total [^{3}H]DPCPX binding was defined as that which occurred in the presence of 5 nM [^{3}H]DPCPX, 0.01% CHAPS and 5.5 U/assay adenosine deaminase. Non-specific binding was defined as the amount of [^{3}H]DPCPX that remained bound in the presence of 2-chloroadenosine (100 μM).

Autoradiography

The autoradiographical technique used was modified from that described by Young and Kuhar (1979). Coverslips were prepared by dipping into Kodak NTB2 emulsion (diluted 1:1 with distilled water; 43°C) and stored over desiccant, protected from light, for 12-48 hr. The emulsion-coated coverslips were glued at one end to slides containing tissue sections previously exposed to [^{3}H]NBMPR or [^{3}H]DPCPX as described above. The slides and coverslips were held tightly together with squares of teflon and binder clips and kept at 4°C for 2-3 ([^{3}H]NBMPR) or 5-6 ([^{3}H]DPCPX) weeks.

To develop the coverslips the binder clips and teflon squares were removed, and the coverslips were gently propped up from the tissue sections. The emulsions were developed in Dektol Developer (diluted 1:1 with water) for 2 min, then rinsed in water for 10 sec, fixed in Kodak Fixer for 5 min

and washed in water for 5 min. The sections were then stained with hematoxylin and eosin and the coverslips were permanently mounted. With this technique correct alignment of the emulsion with the tissue sections was maintained.

Materials

Compounds and radioligands for these studies were obtained from the following sources: [^{3}H]nitrobenzyl-thioinosine (sp. act. 37 Ci/mmol), from Moravek Biochemicals, CA; 8-cyclopentyl-1,3-[^{3}H]dipropylxanthine (sp. act. 120 Ci/mmol), from New England Nuclear, Boston, MA; rabbit polyclonal anti-human von Willebrand Factor, biotinylated anti-rabbit IgG and horseradish peroxidase avidin D, from Dimension Laboritories Inc., Mississauga, Ont.; dipyridamole, diaminobenzidine, CHAPS (3-[(3-cholamidopropyl) dimethyl-ammonio]-1-propanesulfonate), and adenosine deaminase type VII, from Sigma Chemical Co., St. Louis, MO; 2-chloroadenosine, from Research Biochemicals Inc., Natick, MA; glue (Super Bonder 495), from Loctite Corporation, Newington, CT; Kodak NTB2 nuclear track emulsion, Dektol Developer and Kodak Fixer, from Calgary Photo, Edmonton, Alta.; coverslips (25 X 60 mm, Corning No. 1), from Fisher Scientific, Edmonton, Alta.

C. RESULTS

Acetylcholinesterase Histochemistry

Conduction cells, cholinergic nerves and cholinergic ganglion cells were clearly identified by positive staining

for AChE (Fig. 5.1A, 5.2A, 5.4A, 5.5A). Conduction cells, including atrioventricular node, His bundle and left and right bundle branches, were present in approximately 100 sections. The atrioventricular node was often not clearly evident due to the difficulty in obtaining the optimal plane of sectioning. The His bundle was clearly identified in 70% or more of positively staining sections depending on the plane of sectioning.

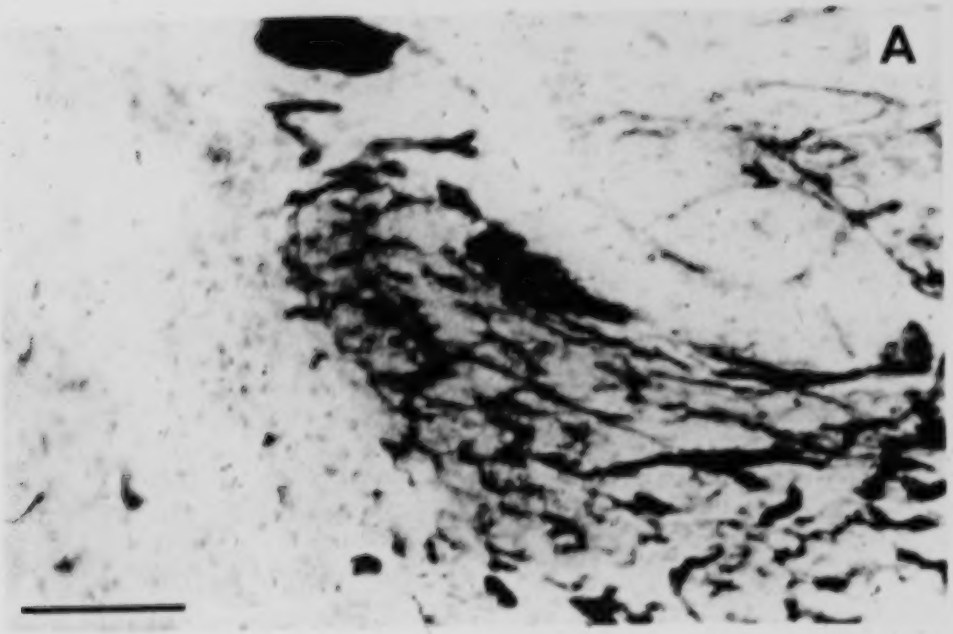
Von Willebrand Factor Immunohistochemistry

Immunoperoxidase staining for von Willebrand Factor was positive for endothelial cells of blood vessels of all sizes and for the endocardial lining of heart (Fig. 5.3C,F,I).

[^{3}H]NBMPR Binding

The distribution of specific [^{3}H]NBMPR binding sites, represented by the difference in distributions of silver grains between total (Fig. 5.1C, 5.2C) and non-specific (Fig. 5.1E, 5.2E) binding autoradiograms, was similar over atrioventricular conduction cells, identified in adjacent sections by heavy staining for AChE (Fig. 5.1A, 5.2A), and cardiac myocytes. In contrast, it is clear that endothelial cells, identified by immunoperoxidase staining for von Willebrand Factor (Fig. 5.3C,F,I), have a much greater grain density, and hence [^{3}H]NBMPR binding site density (Fig. 5.3B,E,H), than cardiac myocytes. The high density [^{3}H]NBMPR binding sites appear to be associated with endothelial cells of the endocardium, venules, arterioles, and the aorta, but are not associated with aortic smooth muscle cells,

FIGURE 5.1: Autoradiographical localization of [^{3}H]NBMPR binding to atrioventricular conduction cells from guinea pig. A, a photomicrograph of conduction cells, identified by dark staining for AChE. An adjacent section stained for hematoxylin and eosin is shown in B. A dark field photomicrograph of the same section, C, shows the distribution of total [^{3}H]NBMPR binding sites. The density of silver grains over conduction cells and ventricular myocytes is similar. An adjacent section, stained for hematoxylin and eosin is shown in D. This section was used to determine the distribution of silver grains in the non-specific [^{3}H]NBMPR binding condition and the dark field photomicrograph is shown in E. A uniform distribution of silver grains is evident over the two cell types. Bar represents 100 μm.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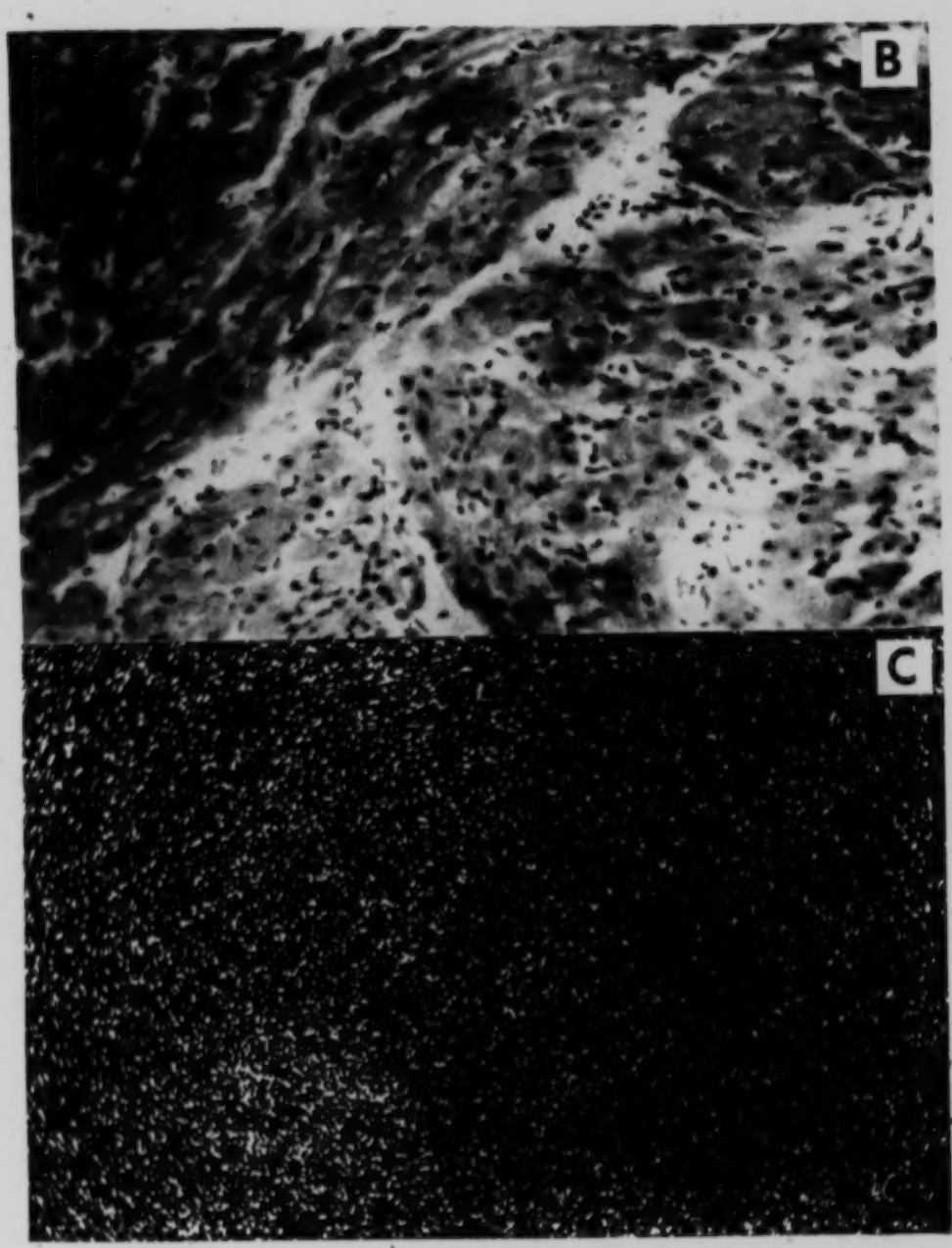
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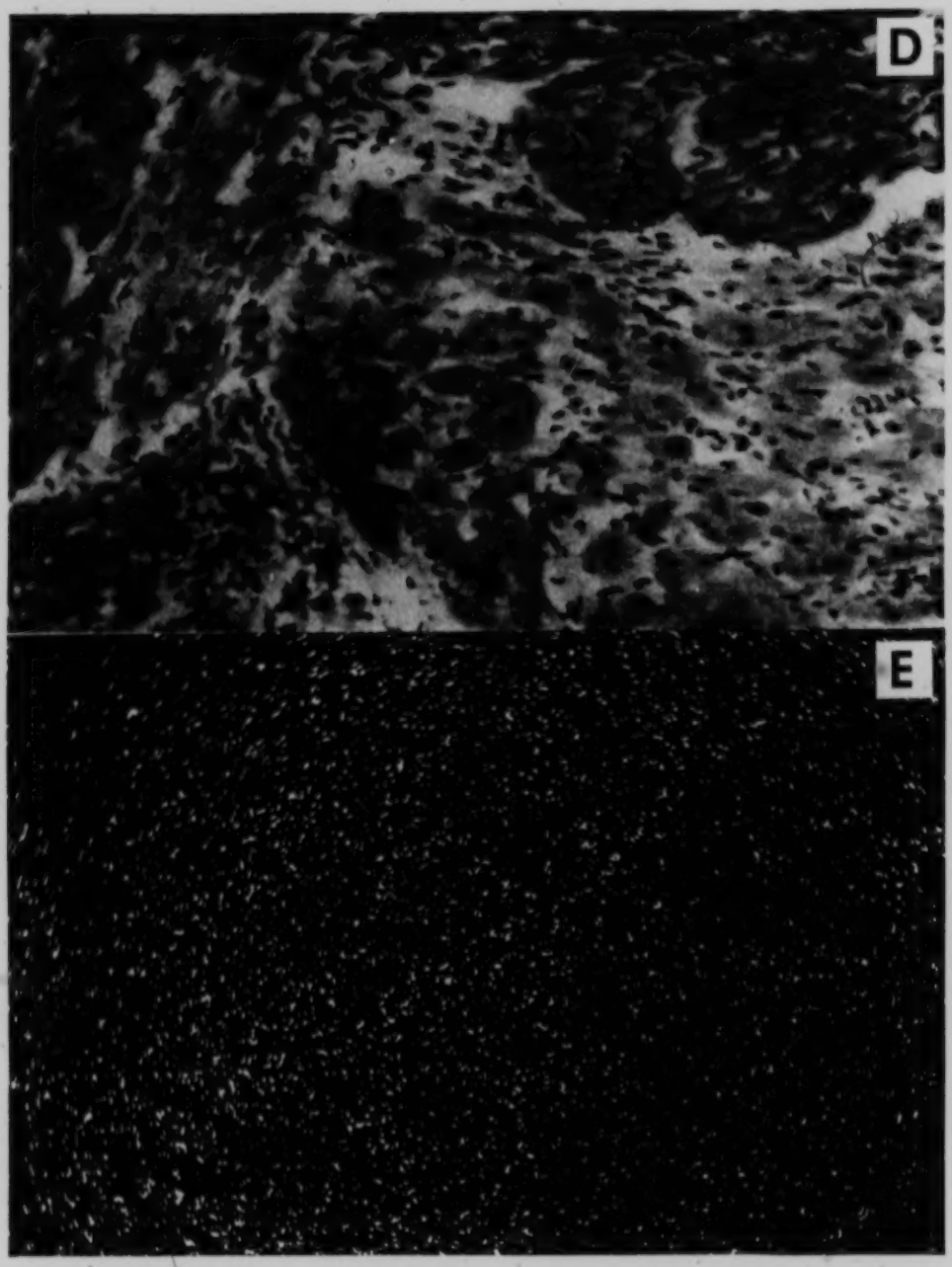
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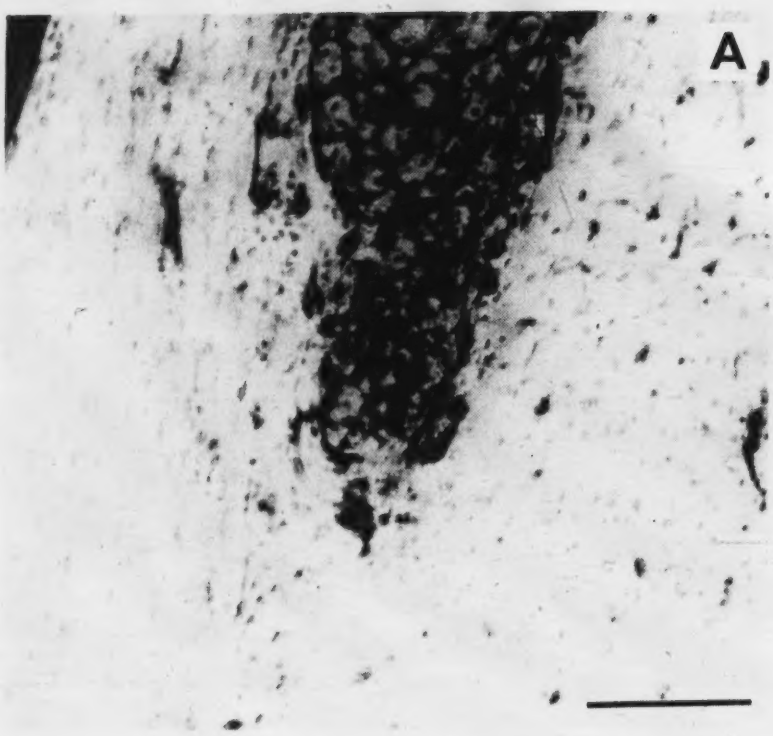
E

FIGURE 5.2: Autoradiographical localization of [^{3}H]NBMPR binding sites in cardiac conduction cells from guinea pig. This figure is similar to Figure 5.1 but the sections were obtained from a different animal. For details see legend for Fig. 5.1.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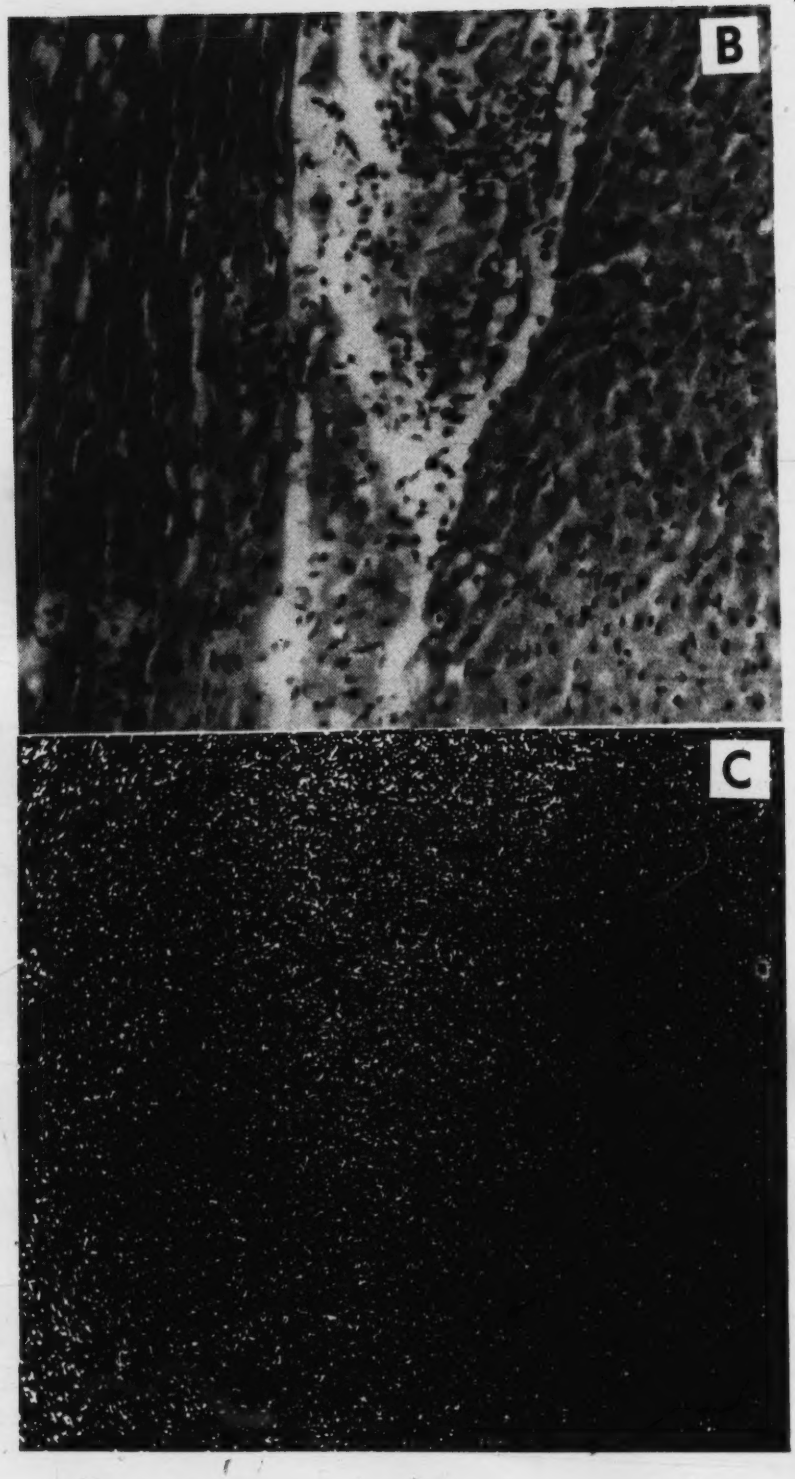
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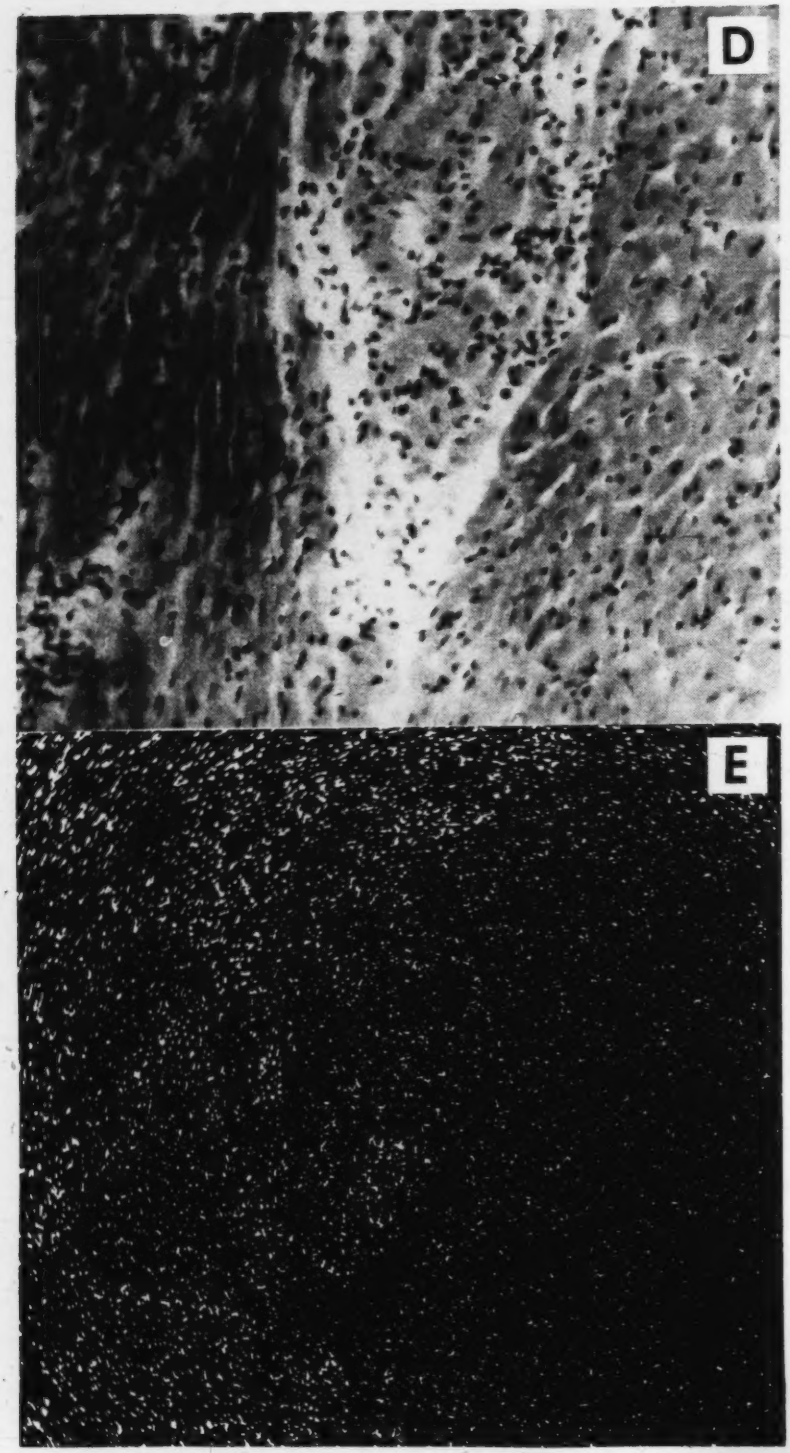
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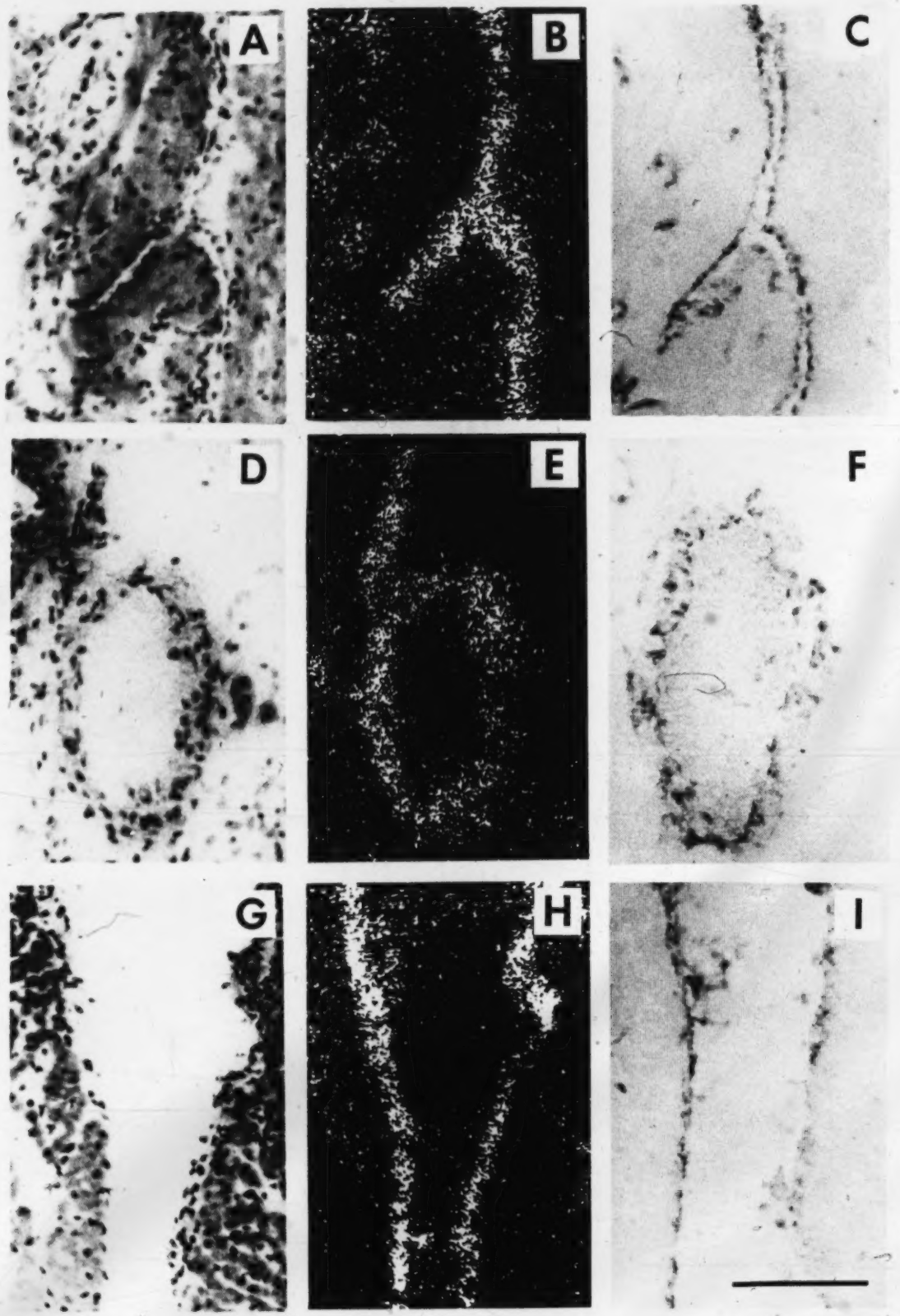
FIGURE 5.3: Autoradiographical localization of [^{3}H]NBMPR binding to cardiac endothelial cells. Bright field photomicrographs of sections stained with hematoxylin and eosin show a blood vessel cut in longitudinal section (A), a blood vessel cut in cross section (D), or endocardial lining (G). Dark field photomicrographs of the same sections show the distributions of total [^{3}H]NBMPR binding sites associated with these structures (B, E, H). Adjacent sections were used to identify endothelial cells using immunoperoxidase staining for von Willebrand Factor (C, F, I). Endothelial cells associated with these structures have high densities of [^{3}H]NBMPR binding sites. Sections used to determine non-specific binding showed uniform grain distributions. Bar represents 100 μm.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cholinergic nerves, or adipose.

[^{3}H]DPCPX Binding

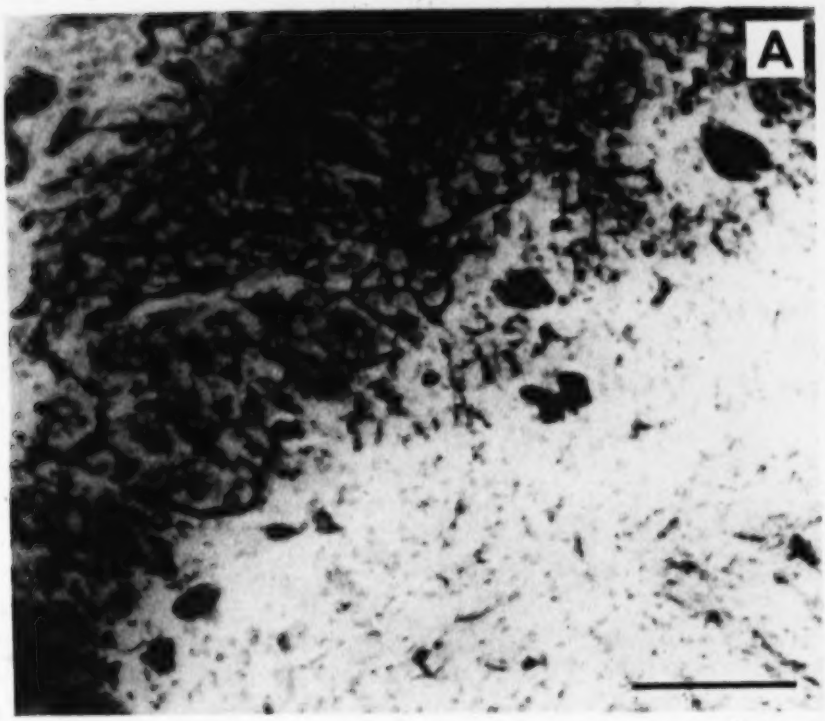
The distribution of silver grains in the total binding autoradiograms (Fig. 5.4C, 5.5C) is non-uniform in contrast to their distribution in the non-specific binding condition (Fig.5.4E, 5.5E). The density of [^{3}H]DPCPX binding sites is greater in conduction cells, identified by dark staining regions for AChE in adjacent sections (Fig. 5.4A, 5.5A), than in cardiac myocytes (Fig. 5.4C, 5.5C). The highest density of binding sites for the adenosine receptor antagonist was associated with conduction cells; no other areas of high binding site density were observed.

D. DISCUSSION

The main finding of this study is that a higher density of adenosine A_1 receptors exists on atrioventricular conduction cells than on cardiac myocytes. Additionally, while there is no difference in nucleoside transporter density between these cells, endothelial cells do have a high density of [^{3}H]NBMPR binding sites. The latter finding agrees with our previous low resolution Ultrofilm [^{3}H] studies (Parkinson and Clanachan, 1989a,b).

Adenosine acts on sinoatrial and atrioventricular cells to produce negative chronotropic and dromotropic effects. The receptors are of the A_1 subtype and are linked by guanine nucleotide binding proteins to potassium channels (Clemo and

FIGURE 5.4: Autoradiographical localization of [^{3}H]DPCPX binding sites in atrioventricular conduction cells from guinea pig heart. A, a photomicrograph of a section stained for acetylcholinesterase; darkly stained cells are conduction cells. An adjacent section, stained with hematoxylin and eosin, is shown in B. This section was used to determine the distribution of the total [^{3}H]DPCPX binding sites and the dark field photomicrograph of this section is shown in C. The grain density is greater over the conduction cells than over ventricular myocytes. An adjacent section, used for the non-specific [^{3}H]DPCPX binding condition, is shown stained with hematoxylin and eosin in D. The dark field photomicrograph of this section is shown in E. The grain density is uniform over conduction cells and ventricular myocytes. Bar represents 100 μm.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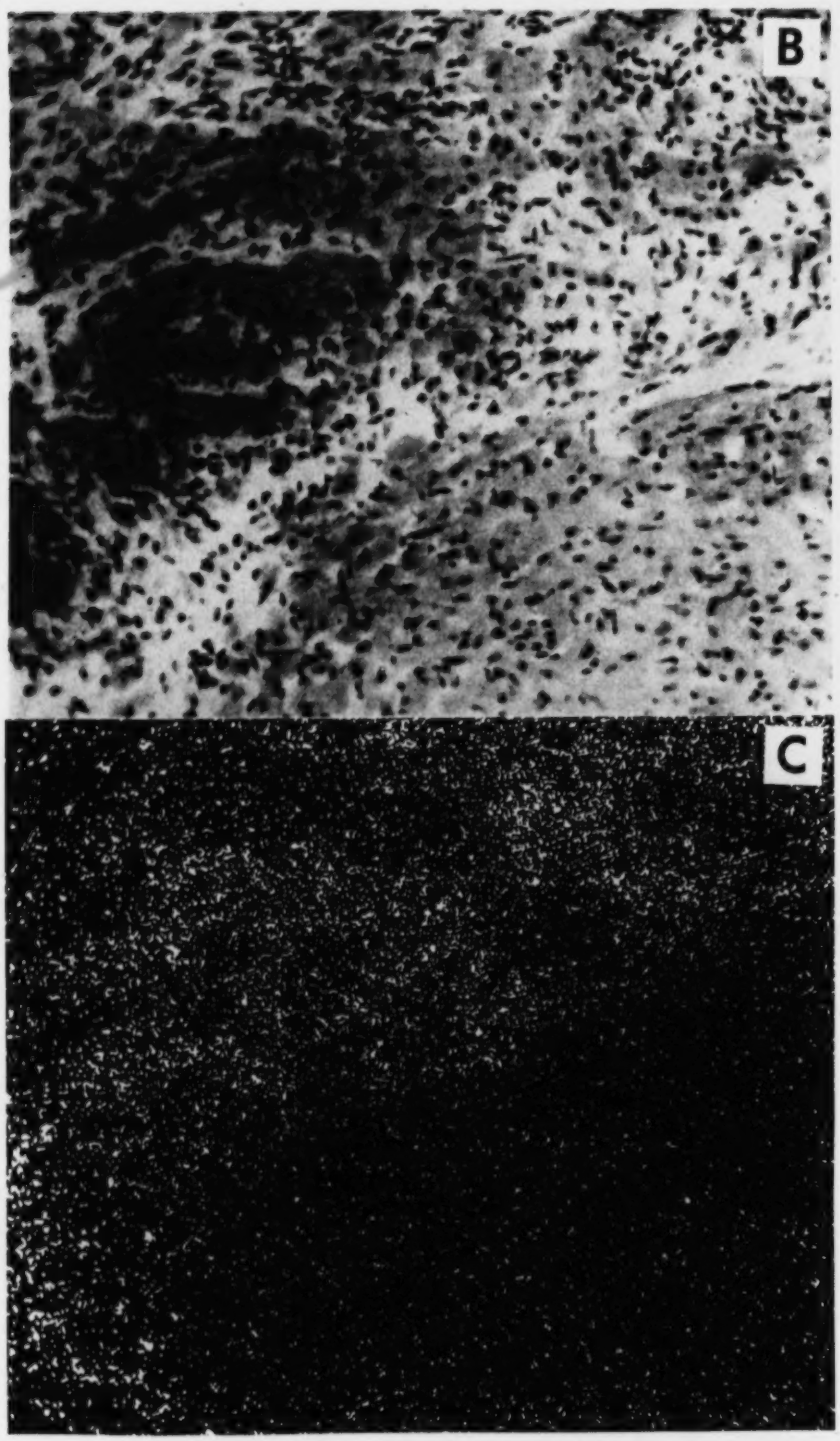
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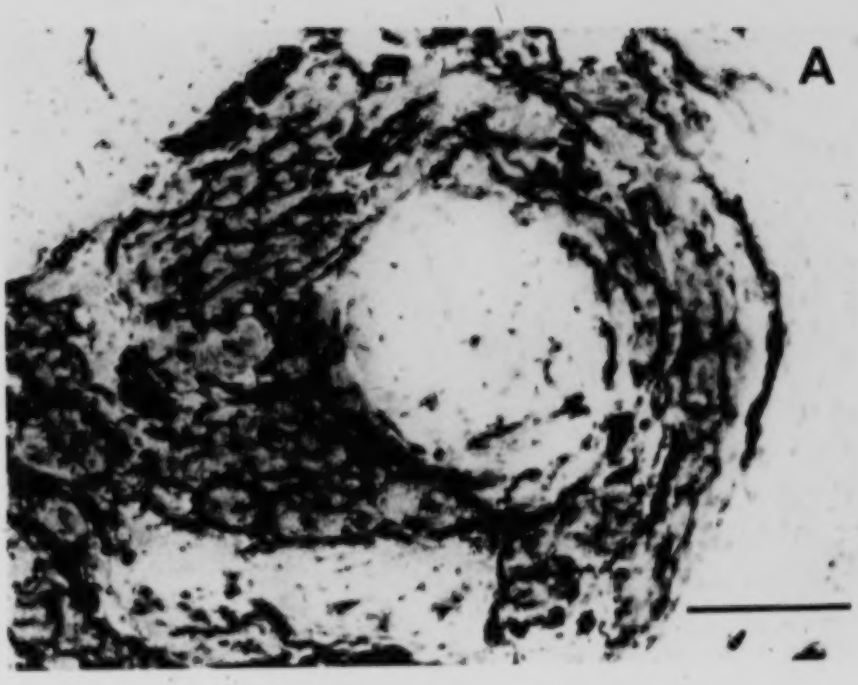
FIGURE 5.5: Autoradiographical localization of [^{3}H]DPCPX binding sites in cardiac conduction cells from guinea pig. This figure is similar to Figure 5.4 but the sections were obtained from a different animal. For details see legend for Fig. 5.4.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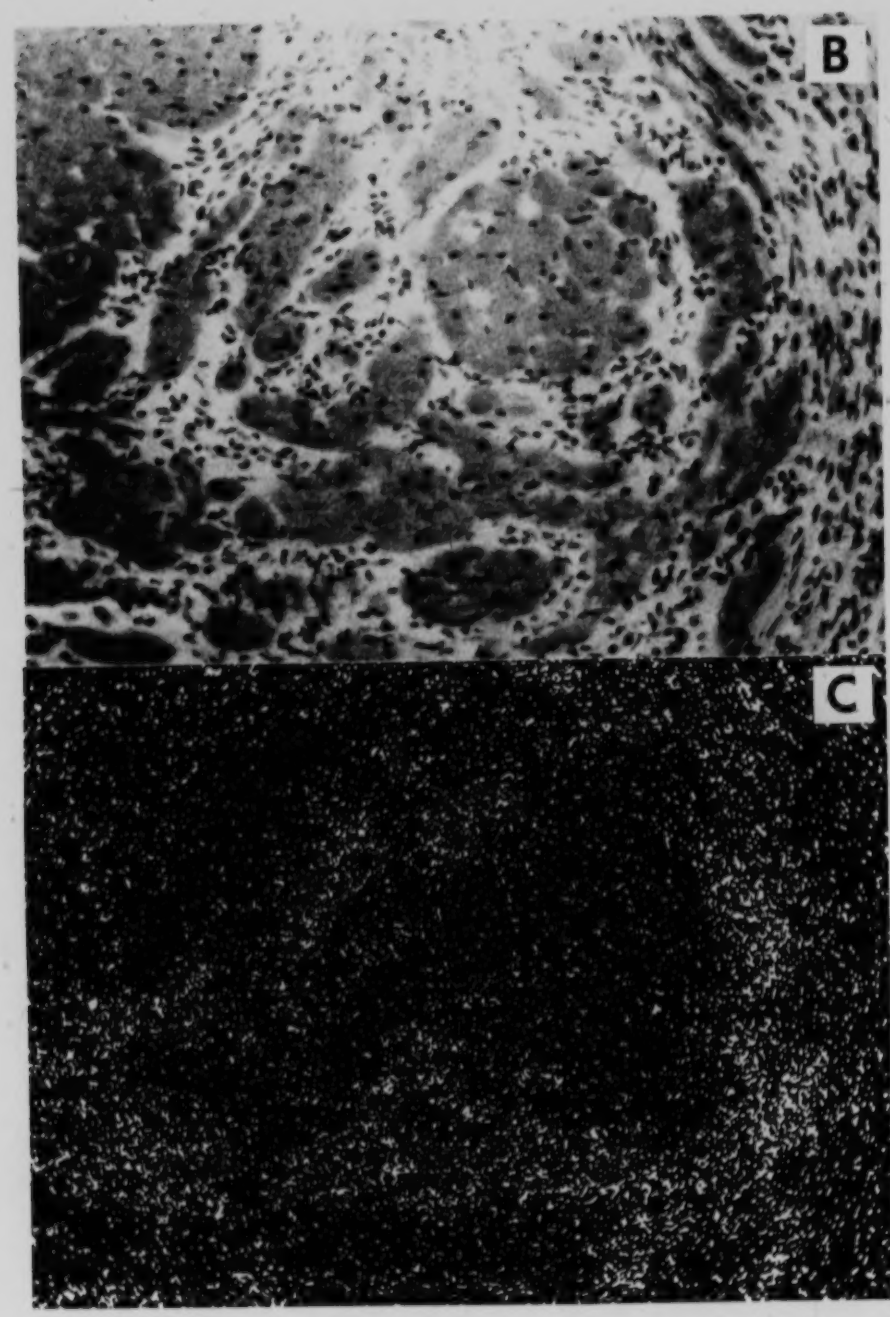
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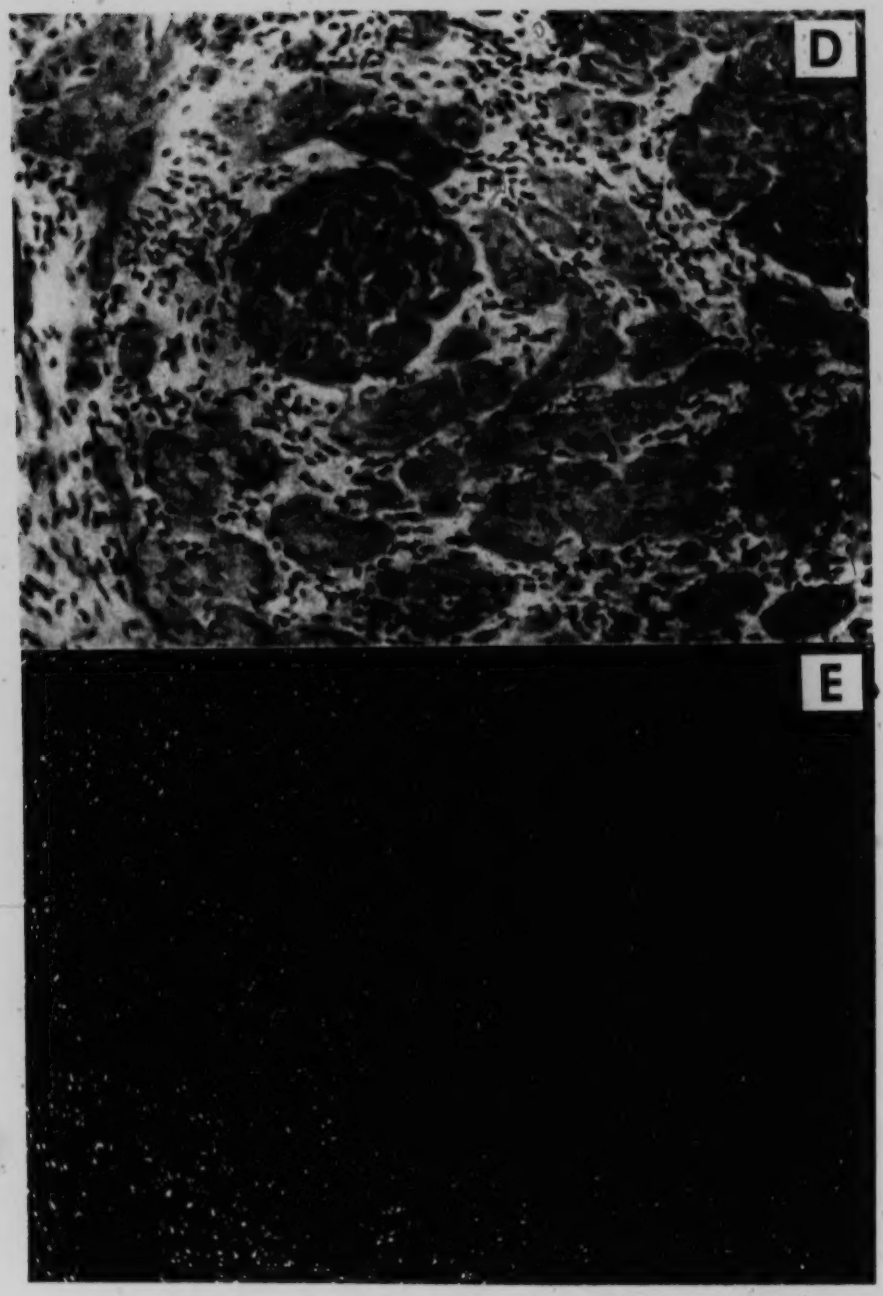
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Belardinelli, 1986a; Bohm et al., 1986). Receptor stimulation leads to increased potassium conductance and hyperpolarization of the cells (Sperelakis, 1987). For the localization of adenosine receptors in this study, the radioligand [^{3}H]DPCPX was used as it is considered relatively selective for adenosine A_1 receptors. It has approximately 500 fold greater affinity for A_1 receptors than for A_2 receptors (Lee and Reddington, 1986; Lohse et al., 1987; Bruns et al., 1987). At the concentration of [^{3}H]DPCPX used (5 nM), only A_1 receptor binding was detectable.

Early work by Belardinelli and co-workers (1980) showed that the conduction delay produced by adenosine was specific to the atria - His bundle interval, while the His bundle - ventricles interval was unchanged. Subsequently, Clemo and Belardinelli (1986a) found that the conduction effects of adenosine were predominantly associated with N cells of the atrioventricular node. However our results, primarily with His bundle and branching bundle cells, show these cells to have increased numbers of A_1 receptors in comparison to atrial and ventricular myocytes.

In the last few years, several nucleoside transport systems have been identified (Paterson et al., 1987) and [^{3}H]NBMPR is a useful ligand for the inhibitory site on facilitated diffusion systems of high sensitivity to NBMPR. Transport studies with isolated guinea pig ventricular myocytes demonstrated this system to be a major component of

nucleoside entry (Heaton and Clanachan, 1987). However it is possible that other cell types in heart may have additional transport systems, not recognized by low nanomolar concentrations of NBMPR. We have shown that putative [^{3}H]NBMPR-sensitive transporters are found in the greatest density in endothelial cells associated with coronary vessels and the endocardial lining.

In light of the high density of transporters on endothelial cells, these cells may be an effective barrier for adenosine. They may be important both in protecting cardiac myocytes from the depressant effects of circulating adenosine and in scavenging adenosine that is released from cardiac myocytes during hypoxic stress.

Previous studies have shown that nucleoside transport inhibitors, such as dipyridamole and NBMPR, potentiate the conduction delay produced by adenosine (Belardinelli et al., 1984). From the autoradiographical analysis of NBMPR binding sites it would appear that this is, in part, due to inhibition of adenosine transport into conduction cells. However, given the higher density of binding sites on endothelial cells, it is likely that these cells also have a significant role. Inhibition of adenosine transport into endothelial cells may contribute to the potentiation of adenosine effects on conduction cells that is seen with these compounds.

The results of this study, showing increased A_1 receptor density and uniform nucleoside transporter density, are consistent with increased effects of adenosine in conduction cells versus other cardiac myocytes. However, it is important to recognize that adenosine receptors in heart utilize two different transduction mechanisms: increased potassium conductance in atrial and conduction cells, and attenuation of catecholamine-induced cAMP accumulation in ventricular myocytes (Belardinelli and Isenberg, 1983a; 1983b). Therefore, the increased adenosine-mediated effects may be due to the different transduction mechanisms in conjunction with increased receptor density. Now that conduction cells can be reliably identified, it will be possible to investigate further the basis of adenosine's pronounced effects on these cells using immunoreagents for guanine nucleotide binding proteins and potassium channels.

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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

A. NUCLEOSIDE TRANSPORT IN KIDNEY

Studies of nucleoside transport in renal tissues are limited. Sodium gradient-dependent transport has been described in renal brush border vesicles prepared from proximal tubules of rats (LeHir and Dubach, 1984; 1985a; b; Lee et al., 1988). There is also a report of K^+-gradient dependent transport of uridine in that preparation (Lee et al., 1988). NBMPR did not inhibit the Na^+-dependent transport system (LeHir and Dubach, 1985a;b) and was not tested in the K^+-dependent system. Trimble and Coulson (1984) reported that dipyridamole did not inhibit adenosine uptake into brush border membrane vesicles, but did inhibit adenosine uptake into basolateral membrane vesicles from rat renal cortex. Based on these reports it would appear that inhibitors of facilitated diffusion nucleoside transport systems do not inhibit Na^+-dependent transport in rat renal tissue.

The autoradiographical distribution of NBMPR binding in sections of guinea pig and rat kidney was described in Chapter 2. NBMPR binding sites were present in greater numbers in the renal cortex than in the renal medulla of both guinea pig and rat. In addition high-density NBMPR binding was observed in small round structures of guinea pig renal

cortex. We suggested that these might represent binding to endothelial cells within glomeruli, since we have shown that there is a high density of NBMPR binding sites in coronary endothelial cells of guinea pigs (Chapter 3).

Because adenosine has been proposed as the mediator of tubuloglomerular feedback (Spielman and Thompson, 1982; Osswald, 1984; Arend et al., 1985; Itoh et al., 1985)[5], it is of interest to determine the distribution of adenosine A_1 receptors and nucleoside transport sites in renal tissue.

There are a few reports in the literature of autoradiographical distribution of adenosine receptors in kidney. In human kidney, A_1 receptors appear to be localized

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[5] Infusions of adenosine into renal arteries of dogs, cats, rats, and guinea pigs produces vasoconstriction of afferent arterioles, decreased glomerular filtration rate and inhibition of renin release (Osswald, 1984). These actions are mediated by A_1 receptors (Rossi et al., 1988; Schnermann, 1988). It has been proposed that these actions constitute a negative feedback mechanism to regulate sodium resorption and metabolic activity of kidney (Spielman and Thompson, 1982; Osswald, 1984; Itoh et al., 1985; Arend et al., 1985). High concentrations of sodium in the distal tubule leads to high activity of Na^+/K^+ ATPase in distal tubule epithelial cells to effect Na^+ resorption. Adenosine, resulting from ATP hydrolysis, diffuses from macula densa cells to juxtaglomerular cells. These cells respond to adenosine by producing afferent arteriolar vasoconstriction and inhibition of renin release. This vasoconstriction decreases the glomerular filtration rate which reduces the sodium load in the glomerular filtrate. Inhibition of renin release results in decreased plasma angiotensin II levels, which then leads to reduced aldosterone concentrations and decreased sodium resorption in the distal tubules. The effects of adenosine on renin release, glomerular filtration rate, and afferent arteriolar dilation are blocked by theophylline and potentiated by dipyridamole (Arend et al., 1985).

to glomeruli (Palacios et al., 1987) whereas in guinea pig, they appear to be associated with collecting tubules (Palacios et al., 1987; Weber et al., 1988). According to our results, NBMPR binding sites in rat kidney do not appear to be in high density in cells associated with glomeruli. In guinea pig, NBMPR bound with high density to what appeared to be glomeruli; however it is important to recognize that the resolution of the method used did not allow us to distinguish between the glomeruli and structures close to the glomeruli. Therefore it is possible that macula densa cells of the distal tubule or juxtaglomerular cells of the afferent or efferent arterioles may contribute to the high density of NBMPR binding sites. Since most of the evidence for the role of adenosine in tubuloglomerular feedback was obtained in dogs and rats it would be useful to determine the distribution of A_1 receptors in these species and nucleoside transport sites in dog.

It is interesting to speculate that renal handling of nucleosides appears to be similar to the handling of glucose: Na^+-gradient dependent transport across the brush border membrane and facilitated diffusion across the basolateral membrane, thus allowing vectoral movement of nucleosides from the tubular lumen to the interstitium and the renal venous effluent. There may be two reasons for the efficient resorption of physiological nucleosides from the glomerular filtrate. Firstly, it is important for the body to conserve nucleosides. Secondly, it may be important to resorb

adenosine in the proximal tubule so as to ensure that adenosine does not reach the distal tubule where it might disturb tubuloglomerular feedback mechanisms (LeHir and Dubach, 1984).

B. NBMPR BINDING SITES IN ENDOTHELIAL CELLS OF HEART

Differences in NBMPR binding site distribution were apparent between rat and guinea pig cardiac sections (Chapter 3). Endothelial cells, identified by von Willebrand Factor radioimmunocytochemistry, had twice the density of NBMPR binding sites of cardiac myocytes in guinea pig, but in rat there was no evidence of unequal site density between these cell types.

The adenosine hypothesis for the autoregulation of coronary blood flow (Berne, 1963) proposes that adenosine is released from cardiac myocytes during conditions of high metabolic activity or low oxygen availability. This adenosine is thought to diffuse through the interstitium to interact with receptors on coronary smooth muscle cells and thereby to produce vasodilation. The ability of endothelial cells to produce and take up adenosine adds complicating factors to the adenosine hypothesis.

The role of endothelial cells in the uptake of adenosine during hypoxia or ischemia has been the subject of much research. Many studies have shown significant uptake of adenosine into cultured and non-cultured endothelial cells

from several organs (Gorman et al., 1986; Nees et al., 1985; Sparks et al., 1985; Pearson et al., 1978; 1983; Pearson and Gordon, 1985). These studies indicate a great capacity for adenosine uptake into endothelial cells, but since uptake was measured the relative contributions of transport into cells and subsequent metabolism was not determined.

Many studies have shown that adenosine is continuously released from endothelial cells in culture (Gerlach et al., 1985; Nees et al., 1985; Shryock et al., 1988), however it has been demonstrated that the effect of hypoxia on this release is minimal (Shryock et al., 1988). This suggests that the contribution of adenosine from endothelial cells to hypoxia-induced coronary vasodilation is unimportant (Sparks and Bardenheuer, 1986), although Deussen et al. (1986) found coronary endothelial cells did contribute, but to a lesser degree than cardiac myocytes, to adenosine release during hypoxia. It would appear that endothelial cells are more important in scavenging the adenosine released from myocytes.

Nucleoside transport inhibitors potentiate the cardiovascular effects of adenosine (Williams et al., 1984). Because they reach the heart through the blood, they might be expected to interact initially with endothelial cells. Since these cells have a great capacity for uptake of adenosine, and according to our results, have a high density of binding sites in guinea pig, the interaction of transport inhibitors with endothelial cells would be predicted to have

a substantial effect on interstitial adenosine levels during periods of myocardial adenosine release.

Whether the lack of a high density of NBMPR binding sites on rat endothelial cells is due to a lower transport capacity or the presence of transport systems that are not detected by low nanomolar concentrations of NBMPR has not been determined. The latter is a distinct possibility in light of NBMPR-insensitive transport that is present in rat erythrocytes (Jarvis and Young, 1986).

C. NUCLEOSIDE TRANSPORTER SUBTYPES

Several nucleoside transport inhibitors and substrates were tested to determine their affinities for NBMPR binding sites associated with cardiac myocytes in guinea pig cardiac sections (Chapter 4). All of the compounds tested had higher affinity for sites on endothelial cells; the greatest selectivities were found with lidoflazine (8.2x) and hexobendine (6.3x).

There are many recent reports dealing with subtypes of facilitated diffusion nucleoside transporters, of normal tissue, that can be detected by different affinities for inhibitors. The species differences in affinity for dipyridamole, with guinea pig tissues having higher affinity than rat tissues, are consistent findings (Hopkins and Goldie, 1971; Williams et al., 1984; Clanachan et al., 1987; Lee and Jarvis, 1988a; b).

The ability of four nucleoside transport inhibitors to inhibit uridine transport in erythrocytes and cultured cells from several species was investigated by Plagemann and Woffendin (1988). Some of the differences observed were species related; for example human cells were more sensitive to dipyridamole and dilazep than rat and mouse cells. In addition to species differences, there were also cell type differences. Human Hep-2 cells were relatively resistant to lidoflazine (IC_{50}=2 μM) compared to human erythrocytes and HeLa cells (IC_{50}=12-140 nM), and HeLa cells possessed dilazep-sensitive (IC_{50}=5 nM) and -resistant (IC_{50}=3 μM) uridine transport in equal proportions. These data support our findings of cell differences in affinity for lidoflazine in guinea pig heart.

More evidence of nucleoside transporter subtypes in normal tissue comes from the demonstration of NBMPR-sensitive and -insensitive transport in rat erythrocytes and rat synaptosomes (Plagemann and Wohlhueter, 1985; Jarvis and Young, 1986; Lee and Jarvis, 1988b) and NBMPR-sensitive and -insensitive [^{3}H]adenosine accumulation in guinea pig brain slices (Davies and Hambley, 1986). These two transport components are often not distinguished by dipyridamole (Plagemann and Wohlhueter, 1985; Davies and Hambley, 1986; Lee and Jarvis, 1988b). However, Lee and Jarvis (1988a), using guinea pig synaptosomes, found biphasic inhibition of [^{3}H]adenosine transport by NBMPR, dilazep, and dipyridamole

and no evidence for Na^+-dependent transport. This suggests two facilitated diffusion transport systems, one with high affinity and one with low affinity for these inhibitors. In dissociated brain cells from rat, Johnston and Geiger (1989) reported a small component of [^{3}H]adenosine accumulation (on a time scale appropriate for transport measurements) that was Na^+-dependent. This component was inhibited by NBMPR but not by dilazep or dipyridamole, and is the first report of Na^+-dependent transport that is affected by nucleoside transport inhibitors.

Early work on NBMPR-sensitive and -insensitive transport indicated that dipyridamole inhibited both components equally (Plagemann and Wohlhueter, 1985; Jarvis and Young, 1986; Davis and Hambley, 1986). [^{3}H]Dipyridamole was used to characterize further transport systems (Deckert et al., 1987; 1988; Marangos et al., 1985; Shi and Young, 1986). It was demonstrated that [^{3}H]dipyridamole labels more sites than [^{3}H]NBMPR and that NBMPR has a biphasic profile for inhibition of [^{3}H]dipyridamole (Deckert et al., 1987; 1988). Another study measuring dipyridamole and NBMPR inhibition of [^{3}H]adenosine accumulation in synaptosomes from guinea pig and rat reported that dipyridamole had monophasic profiles while NBMPR had biphasic profiles, requiring micromolar concentrations to produce the same degree of inhibition as dipyridamole (Morgan and Marangos, 1986). These studies have been used to propose that NBMPR recognizes only a proportion

of the transport sites that dipyridamole recognizes (Deckert et al., 1988). However it should be recognized that while K_D values for [^{3}H]dipyridamole in guinea pig range from 2 - 11 nM (Shi and Young, 1986; Marangos et al., 1985; Deckert et al., 1987) the K_i values for inhibiting [^{3}H]adenosine accumulation by dipyridamole were 138 - 250 nM (Morgan and Marangos, 1986). Potential problems with some of the [^{3}H]dipyridamole data include failing to correct the free ligand concentration for binding to pipette tips and assay tubes, and using the inclusion of cold dipyridamole to measure non-specific binding (Marangos et al., 1985; Deckert et al., 1987). Therefore, while the presence of two transporters in CNS with similar sensitivity for dipyridamole, but different sensitivities to NBMPR appears likely, further experiments with transport rather than uptake studies would be useful. Additionally, [^{3}H]dipyridamole binding experiments using several transporter permeants and inhibitors and more accurate estimates of free ligand concentrations are required.

D. CARDIAC CONDUCTION CELLS

We have shown that adenosine A_1 receptors, identified by autoradiographical identification of [^{3}H]DPCPX binding sites, are present in greater density in cells of the atrioventricular conduction system than in ventricular myocytes. In contrast, the density of [^{3}H]NBMPR binding sites, thought to be associated with nucleoside transport

systems, is similar for these two cell types (Chapter 5). The increase in adenosine receptor density and the apparently unchanged adenosine metabolism may account for, or contribute to, the enhanced responsiveness of conduction cells to adenosine.

Adenosine has pronounced depressant effects on cardiac tissue, however many studies have shown that the receptor density is low (Lee et al., 1986; Leid et al., 1988a; b; Linden et al., 1985a; b; Lohse et al., 1985). From our studies it would appear that the A_1 receptors are not uniformly distributed but are in low density on cardiac myocytes and higher density on conduction cells. In contrast, nucleoside transport systems identified by [^{3}H]NBMPR binding in crude membrane preparations, are present in much greater density than adenosine A_1 receptors (Williams et al., 1984). Our studies supported this finding, since a much longer exposure time was required to identify adenosine A_1 receptors than NBMPR binding sites by autoradiography.

Previous studies have reported that the effects of adenosine are not equal in all conduction cells. For example, Drury and Szent-Gyorgyi (1929) found that atrioventricular nodal effects were more prominent in guinea pig while sinoatrial nodal effects were more apparent in dog, cat, and rabbit. Similarly, Clemo and Belardinelli (1986a) studied the effects of adenosine on atrioventricular nodal cells and found that the cells varied in their responses.

These differences may be due to variations in receptor densities, or subtypes of receptors with different affinities for adenosine. It is also possible that these differences are a result of nucleoside transporter subtypes with differing affinity for adenosine. Alternatively, other components of the receptor system, such as transduction mechanisms, may be involved. Due to the difficulty in positively identifying these cell types and the small numbers of cells in each category, it will be challenging to address these possibilities.

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